

**INTERACTIVE EFFECT OF DIETARY FIBRE AND IMMUNE CHALLENGE ON
THREONINE REQUIREMENT AND INTESTINAL BARRIER FUNCTION IN
GROWING PIGS**

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ABSTRACT

High dietary fibre (**DF**) and immune system stimulation (**ISS**) are thought to limit amino acid availability for protein deposition (**PD**) and growth in pigs. Fibre and threonine (**Thr**) may also play an important role in intestinal barrier function. Therefore, this thesis evaluated the independent and combined effects of high DF and immune challenge [*Salmonella typhimurium* and systemic *E. coli* lipopolysaccharide (**LPS**)] on the Thr requirement for PD and growth in pigs, and on the interactive effects of DF, Thr supply and immune challenge on intestinal barrier function. A nitrogen-balance study estimated 0.68% and 0.78% standardized ileal digestible (**SID**) Thr required to maximize PD in pigs fed low fibre (**LF**) and high fibre (**HF**) diets, respectively when systemic ISS was not present. When systemic ISS was present, SID Thr requirement for PD was estimated at 0.76% and 0.72% for pigs fed the LF and HF diets, respectively. Therefore, HF and ISS independently, but not additively, increased the Thr requirement to maximize PD. A subsequent growth performance study using the same HF diet estimated Thr required to maximize average daily gain (**ADG**) at 0.76% and 0.80% SID Thr using the linear and curvilinear breakpoint model respectively. In a third study, supplementing Thr to meet the requirement for HF and systemic ISS, resulted in a numerically lower ADG in the HF-fed and *Salmonella*-challenged pigs, compared to the LF-fed and *Salmonella*-challenged pigs. This suggested that Thr supply to meet HF and ISS was not sufficient to maintain ADG during an enteric immune challenge and therefore, indicates an additive effect of HF and enteric immune challenge on Thr requirement. Finally, systemic ISS increased lactulose recovery in LF fed pigs but not in HF fed pigs, suggesting that feeding HF had a protective effect against loss of intestinal barrier integrity. This effect appears to be partly associated with mucus secretion in the gut, as HF increased fecal mucin output and ileal intestinal goblet cell numbers and tended to increase MUC2 gene expression in the ileum. The non-additive effect of systemic ISS and HF on PD is consistent with the LPS induced loss of barrier function in the LF fed pigs which contributed to increased Thr requirement for PD. Indeed, no loss of barrier function was observed when systemic ISS and HF were combined, hence no further increase in Thr requirement was observed. In contrast, we postulate that an enteric immune challenge and HF diet resulted in a higher magnitude of impact on gut mucosal protein dynamics that exceeded the gut mucosal protein response to the effect of HF alone, resulting in increased Thr utilization to support mucosal protein synthesis and thereby increasing dietary Thr requirement for growth. In summary, results indicate that immune challenge and high DF will increase Thr

requirement for growth, but DF will have beneficial effects on improving intestinal barrier function in pigs.

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DEDICATION

This work is dedicated to Mr. Theophilus L. Wellington.

Thank You for all you have done for me.

*“In heavenly love abiding, no change my heart shall fear.
And safe in such confiding, for nothing, changes here.
The storm may roar without me, my heart may low be laid
But God is round about me, and can I be dismayed?”*

*Wherever He may guide me, no want shall turn me back.
My Shepherd is beside me, and nothing can I lack.
His wisdom ever waking, His sight is never dim.
He knows the way He’s taking, and I will walk with Him*

*Green pastures are before me, which yet I have not seen.
Bright skies will soon be over me, where darkest clouds have been.
My hope I cannot measure, my path to life is free.
My Saviour has my treasure, and He will walk with me.”*

By

Anna L. Waring

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LIST OF ABBREVIATIONS

AA	Amino acid
ADF	Acid detergent fibre
ADFI	Average daily feed intake
ADG	Average daily gain
AIA	Acid insoluble ash
AID	Apparent ileal digestible
ANOVA	Analysis of variance
AOAC	Association of analytical chemists
Arg	Arginine
ATTD	Apparent total tract digestible
AUP	Animal use protocol
BCAA	Branched chained amino acid
BG agar	Brilliant green agar
BPW	Buffered peptone water
BUN	Blood urea nitrogen
BW	Bodyweight
Casp3	Caspase 3
CCAC	Canadian council of animal care
cDNA	Complimentary DNA
CF	Crude fibre
CFU	Colony-forming unit
CLDN-1	Claudin-1
CLDN-4	Claudin-4
CP	Crude protein
Cys	Cysteine
d	Day
DDGS	Distiller dried grains and soluble
DE	Digestible energy
DF	Dietary fibre

DM	Dry matter
DON	Deoxynivalenol
EDTA	Ethylenediaminetetraacetic acid
ETEC	Enterotoxigenic
FAA	Functional amino acid
g	Gram
G: F	Gain: feed
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gln	Glutamine
h	Hour
HCl	Hydrochloric acid
HF	High fibre
HPLC	High-pressure liquid chromatogram
i.m	Intramuscular
IDF	Insoluble dietary fibre
IFN- γ	Interferon-gamma
IgA	Immunoglobulin-a
IgG	Immunoglobulin-g
IgM	Immunoglobulin-m
IL10	Interleukin 10
IL-1 β	Interleukin 1-beta
IL8	Interleukin 8
Ile	Isoleucine
ISS	Immune system stimulation
kg	Kilogram
L	Litre
Leu	Leucine
LF	Low fibre
LPS	Lipopolysaccharide
LSMEANS	Least-square means
Lys	Lysine

m	Meter
ME	Metabolizable energy
Met	Methionine
mg	Milligram
mg	Milligram
min	Minute
MJ	Megajoules
mL	Millilitre
MLN	Mesenteric lymph node
mM	Millimolar
mRNA	Messenger RNA
MUC2	Mucin gene 2
N	Nitrogen
Nal	Nalidixic acid
NDF	Neutral detergent fibre
NE	Net energy
ng	Nano gram
nm	Nanometre
NO	Nitric oxide
Nov+	Novobiocin
NRC	National research council
NS	Not significant
°C	Degree celsius
PD	Protein deposition
pH	Power of hydrogen
Phe	Phenylalanine
PROC NLIN	Procedure nonlinear
PUN	Plasma urea nitrogen
qPCR	Quantitative polymerase chain reaction
R ²	Coefficient of determination
RNA	Ribonucleic acid

RPL19	Ribosomal protein L19
SAA	Sulphur amino acids
SAS	Statistical analysis software
SCFA	Short-chain fatty acid
SD	Standard deviation
SDF	Soluble dietary fibre
SEM	Standard error of mean
SID	Standard ileal digestible
SQ mean	Starting quantity mean
ST	Salmonella typhimurium
STD	Standard
SUP	Supplemental
TDF	Total dietary fibre
TEER	Trans-epithelial electrical resistance
Thr	Threonine
TID	True ileal digestible
TNF- α	Tumor necrosis factor-alpha
Trp	Tryptophan
μ g	Microgram
μ L	Microliter
Val	Valine
VFA	Volatile fatty acid
Vit	Vitamin
w/v	Weight/volume
w/w	Weight/weight
WBC	White blood cell
ZO-1	Zonula occludin-1

CHAPTER 1
GENERAL INTRODUCTION

The use of a single feedstuff is not practical, nor is it possible to meet nutrient requirement in pigs, therefore, it is important to combine different feedstuffs when formulating swine diets. A wide variety of feedstuffs are available to nutritionists for consideration, however, since the goal is to supply pigs with enough nutrients to meet requirements at the least cost, cheaper feedstuffs are prioritized. As a result, the use of feedstuffs from the food and biofuel production industries has increased in recent years (Woyengo et al., 2014). Products from these industries are identified as co-products and include wheat millrun, distiller's dried grains with soluble (**DDGS**), sugar beet pulp, rice bran, and soybean hulls, among others. These feedstuffs notably have high concentrations of fibre, and therefore when used in high quantities will increase the total dietary fibre (**DF**) content of swine diets. Physiologically, DF is not readily digested by endogenous enzymes but can be fermented in the hindgut, contributing to the metabolizable energy supply for pigs (Stein et al., 1999; Lindberg, 2014). The increase in the use of these co-products in formulating swine diets is reflected as an increase in the level of DF in complete swine diets. Therefore, to obtain optimal benefits from these co-products, it is important to understand the chemical and physical characteristics which affect nutrient digestibility and utilization by pigs, as well as the availability of other nutrients (Nyachoti et al., 1997; Stein et al., 1999; Wenk, 2001).

Previous work has demonstrated that increasing DF in swine diets reduces nutrient digestibility and availability for growth (Stein et al., 1999). Specifically, Libao-Mercado et al. (2006) reported a reduced efficiency of dietary threonine (**Thr**) use for protein deposition (**PD**) when high DF was fed. The effect of high DF on Thr has been related to greater endogenous Thr losses as a result of increased mucin secretion (de Lange et al., 1989; Zhu et al., 2005). Mucin is a glycoprotein rich in Thr and secreted in abundance in the intestinal tract but largely not reabsorbed (de Lange et al., 1989). Blank et al. (2012) reported fibre associated Thr losses in their work evaluating the impact of different fibre sources on nitrogen retention in the growing pig. Similarly, Mathai et al. (2016) demonstrated that feeding diets high in neutral detergent fibre (NDF; 20%) to growing pigs increased the Thr:Lys ratio for maximum nitrogen retention and PD. There is a need to define Thr requirements for growing pigs when feeding high DF as well as the possible role of DF in improving gut health and barrier function. Although there is evidence of negative effects of DF on performance of pigs (Montagne et al., 2012), DF has generated considerable research interest due to other perceived positive functions on the gastrointestinal tract and possible role in barrier function (Lallès et al., 2007; Lindberg, 2014). For instance, dietary fibre has also been

associated with improved barrier function through mechanisms involving changes in the microbial composition, which is further associated with the prebiotic effects of fibre (Chen et al., 2013). Dietary fibre also plays a role in improving barrier function through increased secretion of mucus in the intestinal lumen which functions as a physical barrier, preventing pathogen translocation (Zhang et al., 2019).

Poor health due to immune system stimulation (**ISS**) has been widely reported to affect amino acid (**AA**) availability for growth and PD (de Ridder et al., 2012; Litvak et al., 2013a; Jayaraman et al., 2015; Rudar et al., 2016). Specifically, Kim et al. (2012) demonstrated that demand for sulphur amino acids (methionine and cysteine) requirement for growth increased during ISS with *E. coli* lipopolysaccharide (**LPS**). Similarly, Litvak et al. (2013a) also reported the Met:Met+Cys ratio required for maximum PD increased during *E. coli* LPS induced immune challenge compared with unchallenged control pigs. Li et al. (1999) demonstrated that higher Thr (6.8 g/kg) was required to maximize growth performance in vaccinated vs. unvaccinated pigs. In an enteric disease challenge model, Kahindi et al. (2018) demonstrated that weanling pigs required 66% sulphur amino acids:lysine ratio to optimize growth performance contrary to the 55% ratio recommended by NRC (2012) under unchallenged conditions. These observations suggest that AA requirement for optimal growth will increase during periods of disease challenge. In commercial swine production systems, conditions of sub-clinical disease challenge do occur occasionally and therefore will contribute to reduced animal health, welfare and productive performance, which will indirectly increase production costs. Dietary Thr has been reported to play significant roles in immune response, mucus secretion and promoting muscle growth (Wang et al., 2007; Wang et al., 2010; Mao et al., 2014). During periods of limiting dietary Thr, Munasinghe et al. (2017) reported that Thr use for mucin production is conserved at the expense of muscle growth, suggesting that Thr will be limiting for growth and productive functions during periods of high mucus secretion. Similarly, during periods of disease challenge, higher Thr levels have been reported to be required to optimize growth performance (Li et al., 1999; Wang et al., 2007; Wang et al., 2010; Trevisi et al., 2015). Threonine is the second limiting AA in cereal-based diets and therefore, it is important to define Thr required to achieve maximum growth performance taking into consideration the effects of feeding high DF and sub-clinical disease.

Therefore, the objective of this thesis was to investigate the independent and combined effects of feeding high DF and immune stimulation (enteric *Salmonella typhimurium* challenge and systemic *E. coli* lipopolysaccharide injection) on Thr requirement for growth and PD in growing pigs. Further, the mechanisms relating to the effect of DF, Thr and immune challenge on intestinal barrier function are explored.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Since feed cost contributes largely to the production of pork (Niemi et al., 2010), steps need to be taken to reduce feed costs by pursuing alternative feedstuffs. The use of alternative feedstuffs, mainly co-products from the food and biofuel industries (e.g. wheat bran, DDGS, etc.), have increased in swine diets in the last decade. This is because these co-products are relatively cheaper sources of dietary energy, AA, and minerals (Woyengo et al., 2014). Furthermore, the use of co-products indirectly improves the sustainability of animal agriculture by reducing the reliance on feedstuffs of importance for human food production while utilizing the less desirable co-products to produce high-quality animal protein (Nonhebel, 2004). However, the inclusion of these alternate feedstuffs has nutritional consequences, largely due to their high dietary fibre (**DF**) content, reflected as an increase in the DF content of complete swine diets. Although DF has been reported to play a role in the normal physiological function of the gastrointestinal tract (Wenk, 2001), high DF has also been reported to reduce nutrient utilization, increase AA requirement and reduce growth performance (Blank et al., 2012; Mathai et al., 2016).

Recent regulations controlling the use of antibiotics for growth improvement in swine production can be directly associated with increased exposure of pigs to immune stressors which may elicit both clinical and sub-clinical immune challenge. There is evidence that immune challenge adversely affects animal growth performance, but more importantly increases AA requirement for productive functions (Litvak et al., 2013a; Trevisi et al., 2015; Rudar et al., 2016). Under conditions of immune stress, nutrient use for immune response functions will be prioritized (Le Floc'h, et al., 2004). As such, animal growth and performance will be adversely affected and will indirectly increase production cost. Since the goal of a swine production operation is to increase production performance and increase profits at least cost, factors that affect animal performance are critical. There is a need to examine nutritional and management strategies that will help to maintain animal growth performance during periods of immune challenge. This literature review will discuss the effects of feeding high DF on nutrient utilization and animal performance and highlight the impact of immune challenge on nutrient utilization and animal performance. Further, the review will seek to highlight the nutrition-immune system interaction on AA metabolism.

2.2 Dietary Fibre in Swine Nutrition

2.2.1 Classification and Physicochemical Properties of Dietary Fibre

Dietary fibre was initially defined as the portion of feed mainly lignin and polysaccharides that are not digested by enzymes in the digestive tract (Tromwell et al., 1976). This definition was based on concepts in human nutrition and was commonly applied to other non-ruminant animals. Generally, plant carbohydrates are divided into 3 main categories; monosaccharides (i.e., sugars), oligosaccharides and polysaccharides. The polysaccharides are made up of repeating units of 10 or more monosaccharide or disaccharides held together by glycosidic linkages. Polysaccharides are further divided into 2 groups, (1) starch and (2) non-starch polysaccharide (**NSP**). The NSP are made up of cellulose, hemicellulose, pectin, β -glucans, and lignin (Bach Knudsen, 2001) which form the plant cell wall components and defined as DF (**Fig 2.1**). Other components such as oligosaccharides and resistant starches, which exert similar physiological effects as other plant cell wall components, have been included as part of the definition of DF mainly because they are resistant to enzymatic digestion but readily fermented by gut microbes (Kerr and Shurson, 2013; Lindberg, 2014).

The plant cell wall structure is largely composed of cellulose which is formed by a linear β -(1 \rightarrow 4) glycosidic linkages of glucose units (Bach Knudsen, 2001; Bach-Knudsen et al., 2012). Other components of the plant cell wall include hemicelluloses which are a complex matrix of polysaccharides that may include xylose, arabinose, galactose, mannose, glucuronic acid, and β -glucan units (Montagne et al., 2003; Bach-Knudsen et al., 2012). Pectin is another plant cell wall component made up of glucuronic acid units joined by α -(1 \rightarrow 4) glycosidic linkages. Lignin is a phenolic polymer that is not digested or fermented by endogenous enzymes or intestinal bacteria but holds the plant cell wall components (Bach-Knudsen et al., 2012; Metzler and Mosenthin, 2008). The combination of different polysaccharides determines the physical and chemical properties of the DF and characterises the physiological effects on the mammalian gut (Bach Knudsen, 2001). Based on the physicochemical characteristics such hydration properties, viscosity and fermentability of the polymers that make up the polysaccharide, DF can be broadly divided into soluble and insoluble DF fractions (McDougall et al., 1996).

The soluble DF fraction consists mainly of β -glucans, pectin and gums which are characterised by high luminal viscosity and high-water holding capacity thereby slowing gastric movement and reducing nutrient absorption (Rainbird et al., 1984; Bach Knudsen et al., 1993; Bach Knudsen, 2001). Soluble fibre sources include sugar beet pulp and soybean hulls, which contain high levels of pectin (Noblet and Le Goff, 2001). The soluble fibre fractions are highly fermentable by intestinal microbes and, as such are sometimes referred to as fermentable fibre fractions (NRC, 2012). The beneficial effects of fibre are largely related to its fermentability, which has been suggested to improve gut health and barrier function (Lindberg, 2014). The insoluble DF fraction mainly consists of cellulose and hemicellulose and generally exerts effects on the gut by increasing fecal bulk and decreasing digesta transit time (Glitsø et al., 1999; Bach Knudsen, 2001; Noblet and Le Goff, 2001). Unlike the soluble DF, the insoluble DF fractions are not highly fermentable (Jorgensen et al., 1996; Bach Knudsen, 2001). Dietary fibre sources such as wheat straw, wheat bran and oat hulls have high lignin content and therefore classified as high insoluble DF sources (Noblet and Le Goff, 2001).

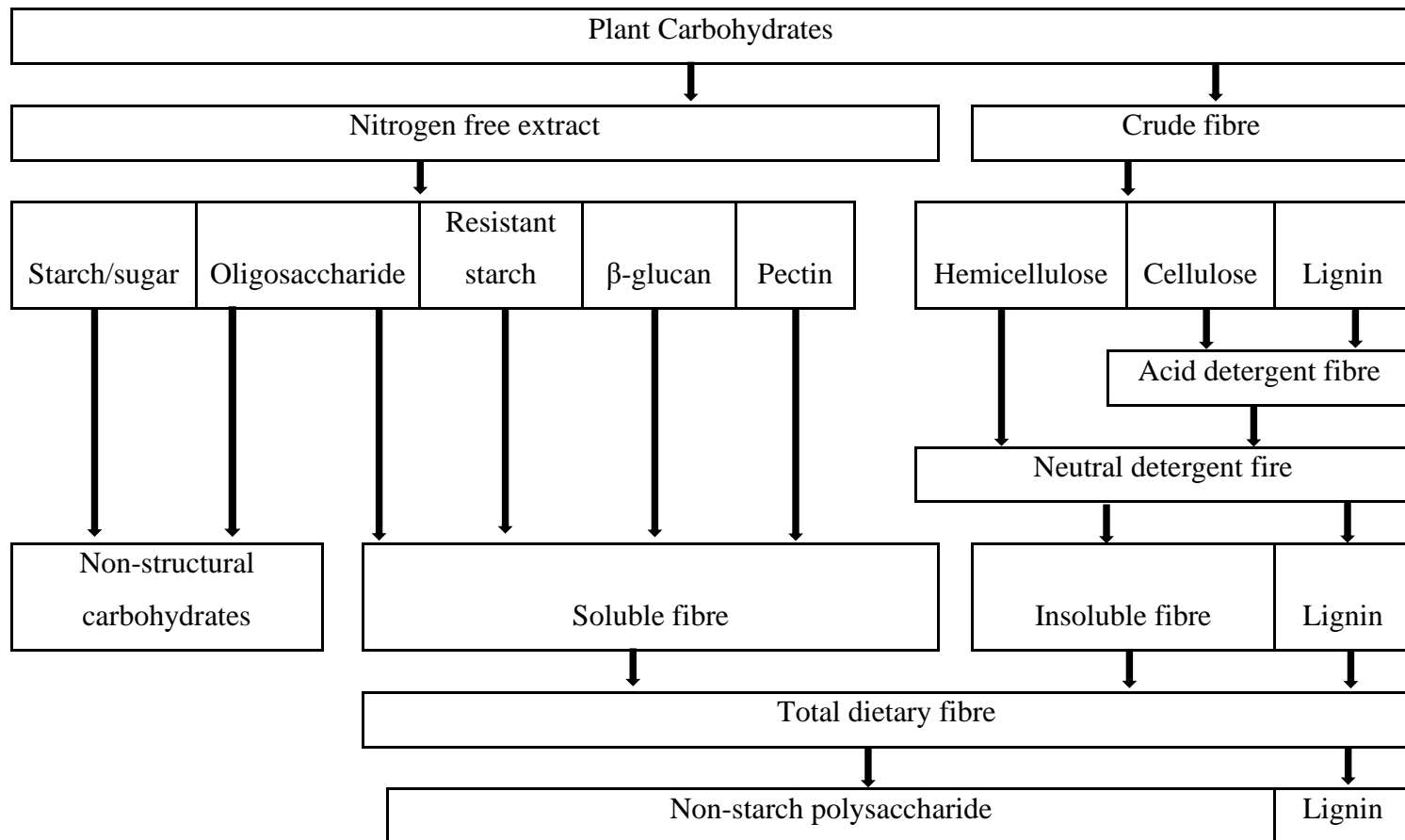


Figure 2.1 Classification of plant carbohydrate fractions (Adapted and modified from Laszlo Degén PhD. Thesis, University of Kaposvar, 2010).

2.2.2 Methods of Fibre Analysis

Numerous analytical methods are available to characterise the DF components in feed, these methods are grouped into chemical-gravimetric, enzymatic-gravimetric and enzymatic-chemical methods (Agyekum and Nyachoti, 2017). Crude fibre (**CF**) analysis is a chemical-gravimetric method first proposed by Henneberg and Stohmann in 1860 as part of the Weende proximate analysis. The analysis is based on residues remaining after acidic and alkaline treatment of feed samples which are meant to imitate animal digestion in the stomach and function of the pancreatic secretions (Mertens, 2003). Although this method of DF analysis has been used extensively, there are issues pertaining to the recovery of some components of DF, such as cellulose, hemicellulose and lignin.

Due to inherent problems with the CF analysis, the detergent fibre methods were developed by Van Soest to classify the DF fractions into the non-digestible cell wall components (i.e., cellulose, hemicellulose and lignin) and the readily digestible cell contents (i.e., starch and sugars). This method is a chemical-gravimetric method which is based on the use of detergents solutions to extract the various components of fibre (Van Soest et al., 1991). The neutral detergent fibre (**NDF**) method is used to recover the cellulose, hemicellulose and lignin fractions, basically the insoluble portions of fibre using neutral pH solutions (Mertens, 2003) while the acid detergent fibre (**ADF**) procedure focuses on extracting the cellulose and lignin fractions by dissolving feed samples in acidic pH solution (Van Soest et al., 1991). The detergent fibre methods are improvements over the CF method but do not fully recover the pectin, gums and β -glucans fractions (Mertens, 2003).

The Englyst and Uppsala tests are both enzymatic-chemical methods which utilize enzymes to hydrolyse the starch component in a fibre sample and use ethanol to precipitate the NSP components of the sample (Mertens, 2003). The methods differ in their quantification of the total fibre content in a sample. While the Uppsala method quantifies total fibre as the sum of amylase enzyme-resistant polysaccharide, lignin and uronic acid; the Englyst method excludes lignin and the enzyme-resistant polysaccharide (Englyst et al., 1996). The total dietary fibre (**TDF**) method was developed to address the drawbacks of the Van Soest detergent methods. The TDF method is an enzymatic-gravimetric method (McCleary, 2003; McCleary et al., 2013) and is currently considered the most accurate method for determining the total DF component in feed

ingredients or complete feed samples. The TDF method includes analyses of both soluble fibre fractions (i.e., oligosaccharides, pectin, resistant starch and β -glucan) and insoluble fibre fractions (i.e., hemicellulose, cellulose and lignin).

2.2.3 Effect of Dietary Fibre on Nutrient Digestibility in Pigs

Energy supply is important for animal body maintenance, production functions (e.g. growth, lactation) and, as such, proper evaluation of energy content of swine feedstuffs is important for maintaining animal performance. The total energy derived after ingesting feed is defined as the gross energy (**GE**), however, in the process of converting GE in a diet into utilizable energy for maintenance and growth purposes, substantial amounts of this GE is lost via feces, urine, gas, and heat production (Noblet et al., 1994). Adjustments to account for these losses result in the calculation of digestible energy (**DE**), metabolizable energy (**ME**), and net energy (**NE**). The main goal of all energy systems is characterizing energy values to feedstuffs such that by feeding pigs a mixture of these feedstuffs the amount of energy supplied to the pig can be estimated and therefore predict animal performance (Noblet and van Milgen, 2004). Indeed, studies have demonstrated the low energy digestibility of DF sources, and therefore increasing the DF content of swine diets will consequently affect energy availability for maintenance and productive functions (Noblet and Le Goff, 2001; Moeser and Van Kempen, 2002; Le Gall et al., 2009). It is also important to consider the composition of the diet, particularly the level, source and type of fibre as these are important indicators that may impact dietary energy. Gross energy digestibility was reported to be higher in finishing pigs fed diets containing sugar beet pulp (80%) as DF source compared to pigs fed diets containing soybean hulls (78%) as DF source (Mroz et al., 2000).

When growing-finishing pigs were fed diets containing either 100 or 200 g/kg DM TDF, a lower apparent total tract digestibility (**ATTD**) of energy was recorded with the 200 g/kg TDF diet (Le Goff et al., 2002). Furthermore, Urriola et al. (2010), evaluated different sources of DDGS to determine their apparent ileal digestibility (**AID**) and ATTD when fed to growing pigs. The result from that study demonstrated that different sources of DDGS had different ileal and total tract digestibility of fibre and further suggested that these differences in ATTD and AID will ultimately affect energy digestibility especially when DDGS sources are combined to formulate a complete

diet. Similarly, Wilfart et al. (2007) reported a progressive decrease in ATTD of energy as the inclusion level of wheat bran increased in the diet of growing pigs. Blank et al. (2012) reported a lower gross energy digestibility (37%) when pigs were supplemented with 300 g/d wheat bran fibre compared to basal diet gross energy digestibility value (86%). Specifically, for every 1% increase in TDF content of a diet, a corresponding 1% decrease in energy digestibility coefficient is expected (Wilfart et al., 2007). The effects of DF on energy digestibility has been attributed to the physicochemical properties (e.g. solubility) of the DF source (Bach Knudsen et al., 1993; Bach Knudsen, 2001). Soluble fibre sources (e.g. sugar beet pulp, pectin) exert their effects on nutrient digestibility by their hydration properties such as solubility and viscosity (Bach Knudsen, 2001; Lindberg, 2014). Insoluble fibre sources (wheat bran, soybean hulls) increase fecal bulk and increase digesta passage rate thereby increasing fecal energy losses (Bach Knudsen, 2001). The effect of fibre on energy digestibility is related to the proportion of absorbed energy-yielding fermentation products (volatile fatty acids) in the hindgut, compared to digestion and absorption of monosaccharides in the small intestine, which generally yield much higher energy (Jorgensen et al., 1996). This reduces the total energy availability in diets containing high DF sources compared to diets lower in fibre.

Crude protein and AA digestibility are essential for increased N retention and growth. Factors that affect N retention include voluntary feed intake, anti-nutritional factors, and dietary protein and fibre level (Souffrant, 2001). High DF was reported to reduce N retention by increasing endogenous N losses (Nyachoti et al., 1997). Earlier studies by de Lange et al. (1989) with protein-free diets demonstrated that adding pectin and cellulose in the diets significantly increased the flow of endogenous N in ileal digesta, which has been confirmed in other studies (Leterme et al., 1998; 2000). Further, Myrie et al. (2008) demonstrated that feeding wheat bran and barley-based diet reduced N retention in growing pigs compared to a casein-based diet with no fibre source. This effect was attributed to a higher ileal flow of protein in the wheat bran- barley-based diets. Also, Galassi et al. (2010) reported a 6% decrease in ileal N digestibility when feeding high DF. The effect of sugar beet pulp, a soluble fibre source containing pectin and glucans on N digestibility was evaluated in growing pigs and the results demonstrated that N digestibility decreased linearly as graded levels of sugar beet pulp were included in a casein-based diet (Zhang et al., 2013a). These studies confirm the effect of DF sources in reducing N retention in pigs by increasing endogenous N losses in ileal digesta. The effect of DF on ileal and total N digestibility is influenced

by the DF source, dietary level and fibre type. Blank et al. (2012) demonstrated this by evaluating different fibre sources on N digestibility in pigs and reported that different fibre sources indeed reduced N digestibility at different rates.

Dietary fibre can also have differing effects on digestibility of individual AA. The apparent ileal N digestibility was reported to be similar in pigs fed high DF compared to pigs fed a low fibre diet. However, ileal digestibility of specific AA was reported to be lower with high DF (Dilger et al., 2004). This effect is related to the physicochemical properties of the fibre, where the solubility of fibre fractions has been described to directly affect ileal AA excretion (Souffrant, 2001). The inclusion of 22.5 or 45% wheat shorts in the corn-starch-based diet reduced AID of most AA (Libao-Mercado et al., 2006). Contrary to this, the addition of cellulose and barley straw to corn-soybean meal-based diets did not reduce AID or SID of AA except for leucine and glycine (Sauer et al., 1991). Similarly, Li et al., (1994) showed that adding cellulose up to about 17% in the diet did not increase endogenous AA losses. This was associated with the fermentability of cellulose (insoluble and poorly fermentable fibre source) and, therefore has limited effects on pancreatic secretion and enzyme activity. These inconsistencies in response to feeding high DF demonstrates that different fibre types will affect N and AA digestibility differently due to their physicochemical properties and specific effects on gut function, nutrient digestibility, and endogenous AA losses.

2.2.4 Effect of Dietary Fibre on Growth Performance of Pigs

The effect of feeding high fibre diets on growth performance of growing pigs has been reported previously, however, results have been inconsistent. In a study with weaner pigs, Emiola et al. (2009) reported an increased average daily gain (**ADG**) in pigs fed high fibre diets (40 g/kg wheat bran and 20 g/kg sugar beet pulp) compared to pigs fed lower fibre diets. Similarly, Hermes et al. (2009), reported greater ADG in high DF (7.5% NDF) fed pigs compared to pigs fed lower DF. The increased ADG observed in this study was related to the increased weight of internal organs, such as the large intestine of high DF fed pigs (Hermes et al., 2009).

In other studies, feeding high fibre diets were reported to have no significant effect on ADG and feed efficiency (Longland et al., 1994; Gill et al., 2000; Galassi et al., 2010). Shriver et al. (2003) reported no significant difference in ADG when diets containing either 10% soybean hull

or 10% dried sugar beet pulp was fed to grow-finish pigs. In contrast, Wellock et al. (2008) reported decreased ADG in weanling piglets fed diets high in NDF (17.7%). Similarly, a linear decrease in ADG was reported when up to 25% of wheat DDGS was added to a wheat-soybean based diet (Thacker, 2006). Feeding diets containing 7% cellulose or guar gum resulted in reduced growth rate and final body weight of growing pigs (Owusu-Asiedu et al., 2006). Growing pigs (25 kg BW) fed increasing levels of corn DDGS up to 30% of total dietary inclusion shows a linear decrease in final body weight (Agyekum et al., 2014).

The dietary energy level and SID AA content are thought to be factors that could reduce growth performance with the inclusion of DF. However, in these studies, diets were adjusted to contain similar NE and SID AA contents suggesting that there may be other factors to consider. The growth performance response of pigs fed high DF will be influenced by age as well as the specific type and level of DF fed. For example, Shi and Noblet (1993) demonstrated an increased efficiency of fibre utilization for energy in sows compared to growing pigs. The mechanism associated with this effect is thought to be the greater hindgut capacity of sows to ferment fibre sources due to higher microbial population present compared to growing pigs.

2.2.5 Effect of Dietary Fibre on Intestinal Health and Barrier Function in Pigs

There is growing evidence that DF plays a role in the physiology and homeostasis of the gastrointestinal tract and can, therefore, modulate intestinal health and barrier function (de Lange et al., 2010; Bach Knudsen et al., 2012). Dietary fibre may modulate gut health through mechanisms involving interactions with components of the gut epithelium as well as microbes present in the gut (Montagne et al., 2003). The effect of DF on gut health and function varies with the fibre type and the dietary levels fed. Feeding high DF can, therefore, be used as a strategy to stimulate intestinal health. For example, direct inclusion of 40 g/kg wheat bran, reduced the *E. coli* population in ileal digesta of weaner pigs compared to control pigs fed diets containing no wheat bran fibre (Molist et al., 2010).

Fermentation of DF sources is a contributing factor to intestinal health and barrier function. The actions of fermentation metabolites such as short-chain fatty acids (**SCFA**) have been reported to influence the intestinal luminal environment and further modulate changes in the intestinal epithelium (Gibson et al., 2004; Chen et al., 2013). These SCFA (acetate, propionate, and butyrate), confer some beneficial effects on intestinal health and function (Bindelle et al., 2008; Molist et al., 2009; Jha and Berrocso, 2015), generally as a result of a reduction in luminal pH which has been shown to inhibit pathogenic bacterial proliferation (May et al., 1994). SCFA can also act as sources of energy for enterocyte proliferation. For example, butyrate has been shown to be a major energy source for colonocytes (Hamer et al., 2008) and stimulates growth and development of the large intestine (Montagne et al., 2004; Hedemann and Bach Knudsen, 2007) as well as increased villi height, crypt depth, and mucosal thickness in the ileum and jejunum of neonatal pigs (Kotunia et al., 2004). Further, butyrate plays modulatory roles on intestinal epithelial function and gene expression via several mechanisms (Cox et al., 1994; Namkung et al., 2011; Canani et al., 2011). For example, butyrate inhibits the nuclear factor kappa B (**NF- κ B**) pathway which further control the expression of genes related to pro-inflammatory cytokines and other immune response proteins (Vinolo et al., 2011). Other mechanisms include the anti-oxidative effect of butyrate demonstrated in humans with ulcerative colitis (Hamer et al., 2009). Some other effects of high DF on intestinal epithelial function, include an increase in goblet cell numbers, increased mucus secretion and increased epithelial cell proliferation (Jin et al., 1994; Saqui-Salces et al., 2017). These changes have been linked to improved nutrient absorption via changes in nutrient receptor function (Jin et al., 1994) and increased mucus secretion leading to improved barrier function as previously demonstrated in pigs and rats (Satchithanandam et al., 1990; Lien et al., 2001; Mariscal-Landing et al., 1995).

2.3 Immune Stimulation Effect on Nutrient Utilization and Growth Performance in Pigs

Immune system stimulation and inflammation are processes that modulate nutrient metabolism in the animal, particularly of AA and energy. During ISS, there is activation of immune cells and secretion of cytokines, which are mainly proteins, to play the crucial role of defence against external stressors (Le Floc'h and Obled, 2004; Newton and Dixit, 2012). Cytokines are molecules produced by immune cells (e.g., dendritic cells, macrophages, lymphocytes) that play

pro- or anti-inflammatory roles in the immune response pathway (Striz et al., 2014). Also, the liver secretes acute phase proteins (e.g., fibrinogen, albumin, haptoglobin) which play regulatory roles in the immune response process (Gruys et al., 1994; 2005).

The energy and AA required to undertake these processes of immune defence are sourced de novo through skeletal muscle catabolism or via dietary AA sources and are directed towards organs (e.g., liver) and tissues involved in the immune response processes (Reeds and Jahoor, 2001; Le Floc'h et al., 2004). Increased circulation of pro-inflammatory cytokines, as a result of an immune stimulation cause changes in AA metabolism, such that protein synthesis is increased in visceral organs at the expense of muscle protein accretion (as reviewed by Le Floc'h et al., 2004). Studies have shown that increased circulating cytokine concentrations negatively impact appetite and voluntary feed intake through regulating activities of the hypothalamus, a mechanism thought to reduce nutrient availability to pathogens during disease situations (Johnson, 1998; Plata-Salamán, 1998). Poor animal performance during ISS can partly be attributed to poor feed intake and the corresponding reduction in energy and AA availability for maintenance and growth functions (Schiavon et al., 2018). These changes in AA metabolism triggered during ISS will generate a specific requirement for AA (Le Floc'h et al., 2004). Therefore, supplementation of AA during immune challenge is thought to conserve muscle protein synthesis while supporting the immune response functions.

There is evidence that AA not only play roles in protein metabolism, relating to protein synthesis and muscle protein accretion, but also during immune system functions (Malmezat et al., 2000; Melchior et al., 2004), and that these roles are prioritized above muscle protein synthesis during ISS (Li et al., 2007). In commercial production systems, in which, sub-clinical immune stressors are present, dietary AA supply will be channelled into mounting an effective immune response reducing availability for muscle PD and resulting in poor growth performance and feed efficiency in growing pigs. Studies have been conducted using different immune challenge models to simulate immune response in pigs in order to quantify the effects of ISS on nutrient requirements. The common models currently include the enteric disease challenge model (*E. coli*, *Salmonella typhimurium* etc.), the non-pathogenic systemic model (bacterial LPS, attenuated vaccine challenge) and the environmental sanitation model (clean vs uncleaned housing or sanitary vs unsanitary systems). These models have been used extensively to quantify nutrient requirements

for maintenance and growth functions in pigs. Results from studies using immune challenge models have consistently shown that, there is reduced growth performance and increase nutrient requirements, however, the specific effects and magnitude of the effects is dependent on the challenge model used. This is indicative of the fact that these models differ in their mode of immune stimulation and, therefore, the use of the appropriate model is critical in getting the right response and as such a more reliable conclusion.

2.3.1 Impact of Immune Stimulation on Growth Performance of Pigs

Low sanitary conditions predispose pigs to immune stressors which may lead to sub-clinical immune challenges and increase maintenance energy and amino acid requirements, negatively affecting growth performance. In a study by van der Meer et al. (2016), pigs kept in low sanitary conditions had lower ADG compared to pigs raised under high sanitary condition, while feed intake remained the same in both groups of pigs. Similarly, ADG was lower in weanling pigs raised under low sanitary conditions compared to those raised under high sanitary conditions (Kahindi et al. 2014).

The mechanism relating to the effects of immune stimulation on reduced growth was evaluated in weanling pigs challenged with LPS. The results show that during immune challenge periods, energy metabolism is shifted towards responding to the immune functions rather than for growth, which results in a higher maintenance energy requirement (Huntley et al., 2018). Similarly, injection of LPS has been shown to decrease feed intake and feed efficiency in weanling pigs (van Heugten et al., 1994; Kang et al., 2014). The immune response of pigs following an enteric pathogen challenge has been reported to negatively affect growth performance, however, the mechanisms involved in responding to an enteric challenge as opposed to a systemic challenge are different (Trevisi et al., 2015; Rudar et al., 2016). Growth performance and feed intake were reported to significantly decrease following *Salmonella typhimurium* challenge (Turner et al., 2002; Burkey et al., 2004; Gebru et al., 2010) a condition which negatively impacted feed intake (Pieper et al., 2012; Knetter et al., 2015).

When weanling pigs were challenged with *E. coli* their weight gain, feed intake and feed efficiency decreased compared to pigs challenged with *E. coli* K88 but received an antibiotic treatment (Wu et al., 2012). Similarly, feed intake, daily weight gain, and AID of N were reported to be lower post-*E. coli* K88 challenge in pigs fed diets supplemented with either spray-dried plasma or hydrolysed casein (Bosi et al., 2001). Capozallo et al. (2017) demonstrated that when pigs were infected with *E. coli* and fed different Trp and SAA levels, higher dietary Trp and SAA improved feed conversion efficiency. Therefore, during an immune challenge, efficient nutrient utilization for growth function is compromised. This is largely based on reduced feed intake, poor nutrient digestibility associated with possible gut damage as shown by Capozallo et al. (2017), which leads to poor AA utilization for PD, largely due to increase in maintenance functions such as tissue repair. Since in commercial production systems, pigs are exposed to these immune stressors, critical nutritional interventions are required to ensure nutrient utilization efficiency in pigs, such that growth performance will not be adversely affected.

2.3.2 Impact of Immune Stimulation on AA Requirements in Pigs

The ideal protein concept, which was originally developed by Mitchel (1962), focused primarily on defining AA requirement to support PD and growth. This concept was advanced by Fuller et al. (1989) to include the estimation of AA requirement for both maintenance and PD functions. The concept currently considers lysine as the first AA limiting growth in pigs, and all other essential AA requirements expressed as a ratio to lysine. In all of this, the focus has only been on the healthy pig without considering changes that may arise due to immune stressors. Amino acid metabolism is affected during immune stimulation (Le Floc'h et al., 2004) due to the role played by AA in the immune response pathway. During immune stimulation, there are physiological changes that occur primarily to mount an effective defence against the foreign antigen. Changes that occur include increased immune cell production, mucus secretion, and cytokine secretion among others (Reeds and Jahoor, 2001). Under conditions of ISS, there is increased utilization of AA for immune functions (Reeds and Jahoor, 2001). Moreover, since nutrient use for immune response is prioritized, growth will be negatively affected during periods of ISS due to reduced AA availability for production functions. Therefore, higher supply of AA may be required to mount an effective immune response while maintaining growth.

Indeed, previous work, using different immune challenge models (non-infectious, infectious and sanitary challenge), has demonstrated increased AA requirement for growth functions. For example, Kahindi et al. (2017) evaluated the requirement for sulphur AA (SAA):Lys ratio in weanling pigs exposed to unsanitary housing conditions and reported that a higher SAA:Lys ratio was required to optimise growth performance. Similarly, Litvak et al. (2013a), evaluated the Met+Cys:Met ratio during immune stimulation induced by *E. coli* LPS and reported that a higher Met+Cys:Met ratio was required to optimize PD compared to unchallenged control pigs. When weanling pigs were inoculated with *E. coli* K88, Trp required to maximize growth was higher compared to unchallenged control pigs (Jayaraman et al., 2017) most likely due to Trp role in the kynurenine pathway (Le Floc'h and Seve, 2007). Under poor sanitary conditions, a higher Thr:Lys ratio (67% SID) was estimated to optimize growth performance compared to pigs raised under high sanitary conditions (Jayaraman et al., 2015). In a recent study, immune stimulation induced by *E. coli* LPS was reported to decrease Lys, Phe and Ile flux, which was attributed to a reduction in whole-body protein synthesis or decreased catabolism of these AA (McGilvray et al., 2019b). There is adequate evidence of nutrition-immune interactions in the literature (Litvak et al., 2013a; Rudar et al., 2016; Huntley et al., 2018) and the observation above highlights the need for higher dietary supply of AA during periods of immune stimulation in pigs to maintain growth (Reeds and Jahoor, 2001; Gabler and Spurlock, 2008).

2.4 Functional AA in Swine Nutrition and Health

The concept of functional AA (FAA) is based on the fact that AA are not only used for muscle protein synthesis, but for other physiological processes that are critical for animal maintenance and production (Wu, 2009). Indeed, there is evidence that some AA classified as nutritionally non-essential play significant roles in the immune response, cell signalling and proliferation, and regulation of gene expression (Kim and Wu, 2004; Mateo et al., 2007; Wu et al., 2010). Other AA which are nutritionally classified as essential have also been reported to play beneficial roles in pigs beyond supporting growth. For example, Thr has been shown to be critical for mucus secretion for improved gut immunity (Munashinge et al., 2017). Therefore, the classification of AA based on dietary requirement for the functions of protein accretion and growth may not be appropriate.

Of key consideration in this review is evidence to support the AA effects on the immune response (Grimble et al., 1992; Li et al., 1999; Le Floc'h et al., 2008). Adequate intake of SAA (Met+Cys) is critical for skeletal muscle protein synthesis but also important for the synthesis of proteins involved in the immune response process (Grimble, 2006). Involvement of Met in glutathione synthesis is reported to be essential for the activation of immune cells (e.g., T-lymphocytes) and secretion of cytokines (Wu et al., 2004). Similarly, Cys plays regulatory roles involving acute phase protein and glutathione synthesis (Roth, 2007; Lu, 2009; Rakhshandeh et al., 2010). Also, SAA has been reported to affect the immune response due to their role as methyl donors for DNA methylation and the subsequent effect on immune cell proliferation (Grimble, 2002). In studies with pigs, LPS induced immune stimulation was reported to increase the requirement for Met+Cys:Met ratio to support PD (Litvak et al., 2013a). Similarly, during immune stimulation period, fractional synthesis rate was lower in pigs fed Met+Cys deficient diet compared to pigs supplied dietary Met+Cys that met requirement (Litvak et al., 2013b).

Branched chained AA (**BCAA**) have been reported to play a significant role in the host immune defence and response pathways (Bauchart-Thevret et al., 2009; Lorin et al., 2014) although significant research into the direct effects of BCAA supplementation on immune function in animal models is lacking. Apart from the well-described role of BCAA in muscle protein synthesis, Leu is specifically reported to stimulate signalling of protein synthesis through activation of the mammalian target of rapamycin (mTOR) pathway (Columbus et al., 2015) and, as such Leu is reported to have more pronounced effect on immune function than Val and Ile (Konashi et al. 2000). Similarly, BCAA has been reported to have effects on cytokine and antibody production (Calder, 2006). *In vivo* studies with mice have shown that feeding diets deficient in BCAA, reduced antibody production and increased susceptibility to *Salmonella typhimurium* (Petro and Bhattacharjee, 1981). In a study with pigs, Ren et al. (2014), fed piglets a protein-restricted diet with or without BCAA supplementation and reported that pigs fed diets supplemented with BCAA had higher jejunal and ileal IgA, IgG and IgM concentration, coupled with improved ileal morphology (villus height and crypt depth).

Glutamine (**Gln**) and arginine (**Arg**) are nutritionally considered non-essential AA, however, due to their reported benefits in metabolic processes other than growth, they have become important to consider for dietary supplementation. Glutamine is reported to be the major substrate for cells involved in the immune system (Wu et al. 1991), such that when extracellular concentrations of Gln was increased to physiological levels in plasma, there was a dose-response effect on increasing lymphocyte proliferation in the rat (Wu et al., 1992). Also, Gln supplementation in weanling pigs decreased apoptosis in enterocytes (Domeneghini et al., 2006) and enhanced proliferation of small intestinal cells (Wang et al., 2008). Arginine (**Arg**) has gained specific research attention due to the reported effects on several physiological functions which includes gastrointestinal health and immune system function (Guo et al., 2010). Many authors have reported the physiological roles played by Arg in enhancing intestinal health and barrier function, but also as a regulator in nitric oxide-mediated metabolism (as reviewed by Li et al., 2007; Wang et al., 2009). Dietary Arg supplementation in rats has been reported to improve intestinal barrier function by increasing cell proliferation and reducing mucosal injury (Sukhotnik et al., 2004). In weanling pigs, supplementation of Arg increased vascular development and reduced weaning stress (Zhan et al., 2008).

In an LPS challenge model, the effect of supplemental Arg on mucosal barrier function was evaluated in weaned pigs. The results demonstrate that Arg supplementation increased secreting IgA, CD4+ and CD8+ T cells in the ileum. Further, there was a decrease in mast cell numbers and decreased lymphocyte apoptosis with Arg supplementation (Zhu et al., 2012). These results have therefore indicated that Arg supplementation may enhance intestinal barrier function and immunity (Wu, 2010). Studies in both *in vivo* and *in vitro* models have shown evidence of the regulatory roles of these FAA on intestinal immunity and barrier function (Wu, 2010). Since the gut plays a significant role in nutrient absorption and barrier function for improved gut health, it is becoming increasingly important to find ways of improving gut integrity and function, and FAA are proving to significant in maintaining gut health and barrier function.

2.4.1 Threonine as a Functional Amino Acid

Threonine is usually the second limiting AA in cereal-based swine diets (Lewis, 2001) after lysine and therefore receives considerable attention. The growth performance and PD response of pigs fed diets with adequate or higher dietary Thr has been well documented (NRC, 2012). Further to this, Thr plays a key role in mucin secretion in the gut (Li et al., 2007; Ruth and Field, 2013) which is important in protecting the gut from endogenous enzymes, luminal antigens and microbes, as well as preventing dehydration of the gut epithelium (Toribara et al., 1993). It has been shown that about 60% of dietary Thr is utilized by the gut, mainly for the synthesis of mucosal proteins (Stoll et al. 1998) for enhanced intestinal barrier function (Wang et al., 2009). When dietary Thr was deficient, mucin secretion in the intestinal mucosa was severely impaired in rats (Faure et al., 2005). This is also important in weanling pigs, where Thr requirement for maintenance functions (e.g., mucosal protein synthesis) were reported to be higher than requirement for growth (Fuller et al., 1989). During sepsis, Thr utilization for mucosal protein synthesis increased even further suggesting that inflammatory conditions will increase Thr utilization for mucin synthesis (Faure et al., 2007). It has been suggested that Thr has some direct effects on immune system function. For example, secretory IgA and immunoglobulin (Ig) in both serum and tissue in pigs and poultry have been reported to increase with higher dietary Thr intake (Mao et al., 2014; Chen et al., 2017). With increased dietary Thr, Chen et al. (2017) observed a downregulation of IFN- γ and IL-1 β in the ileum and reduced serum malondialdehyde concentration in broiler chickens, indicating improvement in oxidation status (Chen et al., 2017).

2.4.1.1 Threonine and Intestinal Barrier Function

Mucins are the main component of mucus, a glycoprotein molecule secreted endogenously to lubricate the intestinal mucosa, and which also plays important roles in maintaining intestinal functions and protection from digestion enzymes and acids (Horn et al., 2009). Earlier studies by Lien et al. (1997) feeding a protein-free diet, demonstrated that different AA make up mucin, however, Thr was the highest component (30%). There is evidence which suggests a high rate of Thr utilization in the intestine and this has been related to the incorporation of Thr into mucosal proteins such as mucins (Schaart et al., 2005).

In a study by Law et al. (2007), when neonatal pigs were fed intragastrically with either 0.6 g Thr/kg/d or 0.1g Thr/kg/d, mucin secretion in the proximal colon was higher in the pigs fed higher Thr compared to those fed lower Thr. However, there were no observed differences in mucin secretion when comparing intragastrically fed pigs with those fed parenterally with the same amount of Thr (0.5 g Thr/kg/d parenterally + 0.1 g Thr/kg/d intragastrically). This implies that increasing amount of Thr correlates to increased mucin production irrespective of the mode of Thr supply. In broiler chickens, mucin secretion was reported to be higher in broilers fed higher Thr levels (0.77%) compared to those fed lower levels (0.70%), indicating the increased use of Thr for mucin secretion when Thr is not limiting (Ospina-Rojas et al., 2013). Nichols and Bertolo (2008) demonstrated that luminal threonine supply has effects on mucosal protein synthesis and specifically mucin production. Using a gut loop model and intraluminal flooding dose method, fractional synthesis rates for mucins were reported to be higher in gut loops perfused with 56 mg/g of Thr compared to 0 or 21 mg/g Thr. These results indicate that endogenous synthesis of mucin is sensitive to luminal Thr concentration. In a rat model, restriction of Thr up to 30% of requirement significantly decreased the synthesis of mucins across the intestinal sections (Faure et al., 2005). However, Munasinghe et al. (2017) demonstrated in weanling pigs that, protein synthesis in mucin secreting tissues will be conserved even when dietary Thr is limiting, suggesting that mucosal protein metabolism will be conserved at the expense of muscle accretion.

The role of Thr in maintaining the intestinal gut structure and mucosal barrier integrity has been demonstrated in pigs. Feeding a low Thr diet (6.5 g of Thr/kg of diet) to pigs increased ileal para-cellular permeability compared to pigs fed a balanced Thr diet (9.3 g of Thr/kg of diet), demonstrating the negative effect of Thr deficiency on barrier permeability and function (Hamard et al., 2010). Hamard et al. (2007) evaluated the role of Thr in maintaining gut integrity by feeding a control diet balanced for Thr (9.3 g/kg) and a low Thr diet (6.5 g/kg) to piglets for 2 weeks. In this study, ileal villous hypotrophy was observed in pigs fed the low Thr diets, demonstrating the need for high dietary Thr to maintain ileal structure. Wang et al. (2010), fed 4 graded level of true ileal digestible (**TID**) Thr to weanling pigs (6-7 kg) and reported distorted villus architecture in the duodenum of pigs fed the lowest dietary Thr level (0.37% TID), also expression of MUC2 genes in the duodenum and jejunum were significantly lower in the 0.37% TID fed pigs. Similarly, Law et al. (2007) found that villus height in piglet receiving Thr deficient diet decreased compared to those receiving the Thr adequate diet. In chickens, Moghaddam et al. (2011) reported increased

villi height, crypt depth and villus surface as Thr increased from 0.80% to 0.87% of total dietary inclusion, however as Thr increased from 0.97% to 1.01%, villi height, crypt depth and villus surface decreased indicating the adverse effect of oversupplying Thr. Altogether, available data indicates that a Thr plays an important role in modulating the development and function of the intestinal epithelium for enhanced nutrient absorption and barrier function. Threonine deficiency will have adverse effects on intestinal epithelial development and reduce intestinal barrier activities predisposing pigs to mucosal infections.

2.4.1.2 Threonine and Immune Function

The role of Thr in modulating immune function in pigs has not been clearly elucidated, although there is some evidence supporting this assertion. In earlier cell culture studies, adding 2 mM Thr to the cell culture medium was reported to inhibit apoptosis, but stimulated cell growth and the production of antibodies (Duval et al., 1991). Some work completed in weanling pigs demonstrated increase serum IgG concentration with greater dietary Thr intake (Wang et al., 2006; Mao et al., 2014). Similar results have been reported by Azzam et al. (2011a), who fed laying hens increasing supplemental dietary Thr and recorded a linear increase in serum IgG concentration. Serum IgG concentration is an indicator of high mucosal immune activity, where IgG functions through a combination of complement-dependent, opsonisation and phagocytic mechanisms to prevent a microbial invasion of the gut (Kochi et al., 1993; Shapiro et al., 2002). Secretory IgA content in ileum and jejunum tissue samples have been reported to increase at a higher Thr inclusion at 3 g/kg of the diets fed to broiler chickens compared to a lower inclusion level of 1 g/kg of the diet (Chen et al., 2017). Increasing dietary Thr has also been reported to attenuate the increase in interferon-gamma (**IFN- γ**) concentrations in serum after *pseudorabies* vaccine-induced immune challenge in pigs (Mao et al., 2014). This result suggests that increasing dietary Thr levels may play a role in reducing inflammation by suppressing the release of inflammatory cytokines, such as IFN- γ . Similar effects of dietary Thr on IFN- γ expression has been reported in broiler chickens challenged with coccidiosis (Wils-Plotz et al., 2013). In this study, broiler chickens challenged with coccidiosis and fed higher Thr (5.3 g of Thr/kg of diet) had reduced expression of IFN- γ in cecal tonsil compared to challenged chickens fed a lower Thr diet (1.8 g of Thr/kg of diet).

In their study, Chen et al. (2017) reported that at a higher level of Thr inclusion (3 g/kg of the diet) upregulation of mucin-2 gene (**MUC2**) mRNA expression and downregulation of IFN- γ and IL-1 β in the ileum of broiler chickens were observed. They further reported a reduction in serum malondialdehyde concentration, a marker for oxidation status in broiler chickens. Work in pigs has suggested a positive impact of Thr in immune stimulated pigs. For example, Li et al. (1999), found that higher levels of Thr was required to increase humoral immune response to Bovine Serum Albumin and Swine Fever Attenuated Vaccine challenge than Thr level required to maximize growth rate when no challenge was present. The increase in Thr requirement is largely attributed to an increase in the maintenance requirements by supporting the immune response function as previously demonstrated by Huntley et al. (2018). In an *E. coli* K88ac challenge model in pigs, Ren et al. (2014) observed that the *E. coli* challenge generally reduced ADG and G: F. However regardless of the presence or absence of the challenge, feeding pigs a higher dietary Thr (7.5 and 11.1 g/kg SID) improved ADG and G:F compared to when pigs were fed lower dietary Thr (3.7 g/kg SID).

In summary, the role played by Thr in immunity and barrier function is critical and is conserved even when dietary Thr is limiting (Munashinge et al., 2017). Although the effect of Thr on the intestinal immune response has been demonstrated in pigs and chickens, the exact mechanisms by which Thr exerts its effects have not been clearly explained. However, these findings point to the importance of dietary Thr in supporting immune response and function. Therefore, to increase production performance under immune challenge conditions, higher Thr levels will be important to meet requirements for supporting both immune response functions and for muscle accretion.

CHAPTER 3
RESEARCH RATIONALE HYPOTHESES AND OBJECTIVES

3.1 Research Rationale

The effect of DF on nutrient digestibility and availability has been explored and available data demonstrates the negative effects of DF on AA availability for tissue accretion, especially for Thr. Increasing DF in swine diets has been shown to increase the endogenous Thr losses, reduce Thr availability for growth (Zhu et al. 2005; Libao-Mercado et al., 2006; Blank et al., 2012) and increase the Thr:Lys ratio required for maximum growth performance (Mathai et al., 2016). Some effort has been made to account for the high fibre associated Thr losses, by adjusting Thr requirement based on dietary fermentable fibre level (NRC, 2012). In addition to diet composition, immune status can affect AA requirements for optimal growth performance (de Ridder et al., 2012; Litvak et al., 2013a; Rakhshandeh et al., 2014). Earlier studies by Reeds and Jahoor, (2001) and Reeds et al. (1994) demonstrated that ISS redirects AA away from growth functions to support immune response functions, thereby increasing AA use for maintenance functions and increase requirement for growth. Although high DF has been associated with reduced nutrient digestibility and availability, some positive aspects of fibre are related to its effects on intestinal health and barrier function, including previously demonstrated changes in microbial composition (Chen et al., 2013), effects on intestinal cell proliferation and increased goblet cell numbers (Saqui-Salces et al., 2017). Immune challenge directs changes in intestinal barrier function with considerable overlap in changes in mucus secretion and cell proliferation as noted for DF above. It is, therefore, reasonable to assume interactive effects of dietary fibre and immune challenge. In commercial swine production systems, issues relating to immune challenge in pigs are possible, and with the increasing DF contents of typical swine diets, there is the probability of a combined effect of ISS and DF affecting nutrient utilization and impacting animal performance. Studies focusing on the combined effect of high DF and ISS are lacking. Therefore, the objectives of the studies presented in this thesis were to investigate the independent and combined effects of feeding high DF and ISS on Thr requirement in growing pigs and to characterize the independent and combined effect of DF, dietary Thr and ISS on intestinal health and barrier function.

3.2 Research Hypotheses

1. High DF and immune challenge will additively increase threonine requirement for maximum protein deposition in growing pigs (Chapter 4 and 5).
2. Growth performance of pigs fed high DF and exposed to an enteric pathogen challenge would be maintained when Thr requirement for high DF and systemic immune challenge are met (Chapter 6).
3. High DF will increase mucus secretion and improve intestinal barrier function regardless of the immune status of pigs (Chapter 7).

3.3 Research Objectives

1. To determine the independent and interactive effects of high DF and systemic immune stimulation on threonine requirement for protein deposition (Chapter 4)
2. To determine the optimal threonine requirement to maximize growth performance when growing pigs are fed high DF (Chapter 5).
3. To investigate whether supplementing Thr to meet high DF and systemic immune challenge requirements would maintain growth performance of pigs exposed to an enteric immune challenge when fed high DF (Chapter 6).
4. To characterize the independent and interactive effects of dietary fibre and, dietary threonine on markers of intestinal barrier function and gut health during immune challenge (Chapter 7).

CHAPTER 4

IMPACT OF DIETARY FIBRE AND IMMUNE SYSTEM STIMULATION ON THREONINE REQUIREMENT FOR PROTEIN DEPOSITION IN GROWING PIGS

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Author Contributions

DAC, AVK and JKH conceived and designed the experiment. MOW carried out the experiment analyzed all data. MOW, DAC, AVK drafted the manuscript. All authors read and approved the manuscript.

4.1 Abstract

High dietary fibre (**DF**) and immune system stimulation (**ISS**) are thought to limit amino acid availability for protein deposition (**PD**) in growing pigs. A nitrogen-balance study was conducted to determine Thr requirement for optimal PD when DF and immune system stimulation (**ISS**) were present alone and in combination. A total of 90 barrows (20.5 kg initial BW; SD = 0.75 kg) were randomly assigned to 1 of 10 dietary treatments (n=9) in 9 blocks. Diets consisted of a low fibre (**LF**; 12.5% total dietary fibre) or high fibre (**HF**; 18.5% total dietary fibre by adding 10% sugar beet pulp and 5% wheat bran to the LF diet) with graded levels of Thr (0.49, 0.57, 0.65, 0.73 and 0.81% standardized ileal digestible [**SID**]) fed at $2.2 \times$ maintenance ME requirements. After an 8-d adaptation, two 4-d nitrogen balance collection periods (pre-ISS and ISS) were conducted. Immune system stimulation was induced by repeated intramuscular injection of increasing doses of *E. coli* lipopolysaccharide. Blood samples were taken during both periods to assess acute phase proteins and complete blood analyses. Data were analyzed by PROC MIXED with fixed effects of period, Thr, fibre, and their interactions, with block as a random effect. Threonine requirement was estimated using PROC NLIN quadratic break-point model. The serum concentration of albumin, haptoglobin, fibrinogen, white blood cell and platelet counts were affected by ISS. During pre-ISS, PD increased linearly ($P < 0.01$) as Thr concentration in the diet increased, with a significant interaction ($P < 0.05$) between fibre and Thr. During ISS, PD increased linearly ($P < 0.05$) as Thr concentration in the diet increased. The quadratic break-point model estimated SID Thr required to maximize PD of pigs fed LF and HF diets during the pre-ISS period was 0.68% ($R^2 = 0.88$) and 0.78% ($R^2 = 0.99$), respectively. During ISS, the SID Thr requirement was estimated at 0.76% ($R^2 = 0.76$) for LF diet and 0.72% ($R^2 = 0.95$) for HF-fed pigs. High fibre and ISS independently increased Thr requirement for maximum PD, but these effects were not additive. High DF may, therefore, mask the effects of ISS on Thr requirement for PD.

4.2 Introduction

Protein deposition (**PD**) which represents a balance between synthesis and degradation of proteins, is an important parameter to measure the efficiency of protein utilization for growth (Metayer et al., 2008). To maximize whole body PD, it is necessary to determine dietary amino acid (**AA**) requirements under different environmental and physiological conditions. Current production systems expose pigs to varied environmental and physiological stressors, which may provoke immune stimulation. Immune system stimulation (**ISS**) affects metabolism and utilization of AA by activating immune cells that release cytokines, disrupting normal metabolism and redirecting AA towards supporting immune response rather than growth (Reeds et al., 1994; Le Floch et al., 2004). Threonine is of importance during an immune challenge for the synthesis of acute-phase proteins and immunoglobulins (Reeds and Jahoor, 2001; Li et al., 2007). Furthermore, Thr is abundant in mucins (Munasinghe et al., 2017) which are secreted in response to an immune challenge at mucosal surfaces (Rakhshandeh et al., 2013). Although DF plays an essential role in gut health and function (Lindberg, 2014), studies have reported a reduced efficiency in Thr utilization for growth and PD when pigs are fed diets high in DF (Zhu et al., 2005; Libao-Mercado et al., 2006; Mathai et al., 2016). This effect has been related to increased endogenous protein losses (Jansman et al., 2002; Blank et al., 2012) associated with an increase in intestinal mucous secretion (Myrie et al., 2008). The synthesis of mucous is dependent on Thr abundance, and under conditions of limited dietary Thr supply, protein synthesis will be conserved in mucin-secreting tissues at the expense of muscle growth (Munasinghe et al., 2017). The present study was therefore aimed at quantifying the effect of ISS and high DF on Thr requirement for PD and since both ISS and high DF affect different aspects of Thr utilization, we further hypothesize additive effects when both ISS and high DF are present together.

4.3 Materials and Methods

The experimental protocol was reviewed and approved by the University of Saskatchewan's Animal Research Ethics Board (AUP 20160107) and followed the Canadian Council on Animal Care guidelines (CCAC, 2009).

4.3.1 Animals, Housing, Diets and Experimental Design

A total of 90 growing barrows (Camborough Plus \times C3378: PIC Canada Ltd.) with initial BW of 20.5; SD = 0.75 kg were used in an N-balance study at the Prairie Swine Centre, Inc. (Saskatoon, Canada). Pigs were individually housed in metabolism crates (1.4 m \times 1.5 m) in a temperature-controlled room at 20 ± 2 °C. Pigs were randomly assigned to 1 of 10 dietary treatments over 9 blocks with a total of 10 pigs per block and 9 pigs per treatment. The dietary treatments were arranged as a 2 \times 5 factorial in a randomised complete block with factors of fibre level [high fibre (**HF**) or low fibre (**LF**)] and threonine level (0.49, 0.57, 0.65, 0.73 and 0.81% SID). Wheat-soybean meal-based diets were formulated to meet or exceed nutrient requirements according to NRC (2012) except for Thr. The HF diets were formulated by partly replacing corn in the LF diet with 5% wheat bran and 10% sugar beet pulp (Table 4.1).

The diets were isonitrogenous and isoenergetic and contained celite as an indigestible marker (Emiola et al., 2009; Lan et al., 2008). As ISS has been shown to reduce feed intake, all animals received a restricted intake of $2.2 \times$ maintenance ME requirement (197 kcal/kg BW^{0.60}; NRC, 2012) throughout the study (Litvak et al., 2013a; Rudar et al., 2016). Feed was offered in two equal meals at 0800 h and 1500 h with ad libitum access to water. Feed orts were collected for each pig daily and weighed to determine actual daily feed intake. The experimental period was a total of 16 d in duration and consisted of an 8-d adaptation period followed by two 4-d collection periods, a pre-ISS period and an ISS period. Immune system stimulation was achieved by intramuscular injection of *Escherichia coli* lipopolysaccharide (**LPS**; O55:B5, Sigma Aldrich, Oakville, ON, Canada) at an initial dosage of 30 μ g/kg BW given on d 1 of the ISS period, at least 1 h before the morning meal. A second injection was given 48 h later with the dose increased by 15% to counteract the possibility of tolerance (Rakhshandeh and de Lange, 2012).

Table 4.1 Composition of experimental diets with the highest and lowest threonine (as-fed basis)

<i>Ingredients, %</i>	SID² Threonine, %			
	Low Fibre		High Fibre	
	0.49	0.81	0.49	0.81
Corn	22.0	21.7	4.3	4.0
Barley	7.0	7.0	7.0	7.0
Wheat	48.0	48.0	48.0	48.0
Soybean meal	18.2	18.2	18.5	18.5
Wheat Bran	-	-	5.0	5.0
Sugar beet pulp	-	-	10.0	10.0
Canola oil	1.20	1.20	3.80	3.80
L-Lys -HCl ³	0.58	0.58	0.56	0.56
DL-Met ³	0.20	0.20	0.20	0.20
L-Trp ³	0.04	0.04	0.03	0.03
L-Val ³	0.09	0.09	0.09	0.09
L-Leu ³	-	-	0.07	0.07
L-Thr ³	0.00	0.33	0.00	0.33
Limestone	1.25	1.25	1.05	1.05
Monocalcium phosphate	0.75	0.75	0.71	0.71
Salt	0.20	0.20	0.20	0.20
Vitamin-mineral premix ⁴	0.10	0.10	0.10	0.10
Celite	0.40	0.40	0.40	0.40
<i>Calculated nutrient content⁵</i>				
ME, MJ/kg	13.9	13.9	14.0	14.0
NE, MJ/kg	10.5	10.5	10.5	10.5
CP, % SID	17.3	17.5	17.7	17.9
Thr, % SID	0.49	0.81	0.49	0.81
Lys, % SID	1.12	1.12	1.12	1.12
Met+Cys, % SID	0.68	0.68	0.68	0.68
Val, % SID	0.75	0.75	0.75	0.75
Trp, % SID	0.22	0.22	0.22	0.22
Ile, % SID	0.59	0.59	0.59	0.59
Leu, % SID	1.12	1.12	1.12	1.12

¹The experimental diets with the intermediate Thr (0.57, 0.65 and 0.73 % SID) were prepared by blending the 0.49 and 0.81 % SID diets in appropriate proportions.

²SID = standardized ileal digestible.

³Supplied by Evonik Nutrition & Care GmbH (Hanau-Wolfgang, Germany).

⁴Supplied per kg of complete diet: vitamin A, 8000 IU; vitamin D, 1500 IU; vitamin E, 30 IU; menadione, 2.5 mg; vitamin B12, 0.025 mg; thiamine, 1.00 mg; biotin, 0.10 mg; niacin, 20 mg; riboflavin, 4 mg; pantothenate, 12 mg; folic acid, 0.50 mg; pyridoxine, 2.0 mg; Fe, 100 mg; Zn, 100 mg; Mg, 40 mg; Cu, 15 mg; Se, 0.30 mg; and I, 1mg.

⁵Nutrient content of diets based on nutrient content of feed ingredients according to NRC (2012).

4.3.2 Blood Sampling and Rectal Temperature Measurement

Blood samples were taken from all pigs during pre-ISS and ISS periods 3 h after the morning meal. On the first day of the pre-ISS period, 2 blood samples were collected into 10 mL tubes from each pig via jugular puncture. Similarly, 2 blood samples from each pig were collected on the first day of ISS period, 4 h after LPS injection. The vacutainer collection tubes either contained EDTA or no additive (BD, Vacutainers Mississauga, ON, Canada). Blood samples in EDTA-coated tubes were immediately submitted for complete blood cell and fibrinogen analysis (Prairie Diagnostic Services, Saskatoon, Canada). Samples collected in additive-free tubes were allowed to clot and centrifuged at $2,500 \times g$ at 4 °C for 15 min. Serum samples were collected and stored at -20 °C. Rectal temperature was monitored on d 1 and 3 during both pre-ISS and ISS periods (4 h post-LPS injection during the ISS period and same timeline during the pre-ISS period) using a digital thermometer.

4.3.3 Nitrogen Balance

During each N-balance period, fresh fecal grab samples were collected daily for each pig and stored at -20 °C. At the end of the experiment, fecal samples were thawed, pooled for each pig in each N-balance period and homogenised. Subsamples were taken and stored at -20 °C until analysis. Urine samples were collected quantitatively daily during each N-balance period for each pig using collection jars placed under the metabolism crates for each 24-h period. Urine collection jars contained enough quantities of 6 N HCl to maintain urine pH below 3 to reduce N losses through ammonia volatilization (de Lange et al., 2001). At the end of each 24 h urine collection, total urine was weighed, and a 10% aliquot sampled per pig. All urine samples were pooled for each pig per period and stored at -20 °C until further analysis.

4.3.4 Analytical Procedures

Diet samples were analyzed for AA composition using ion-exchange chromatography with post-column derivatization with ninhydrin (Evonik Nutrition & Care GmbH, Hanau, Germany; Llames and Fontaine, 1994). Fecal samples were dried in a forced-air draft oven at 55 °C for 72 h before grinding in a centrifugal mill (ZM 100, RETSCH GmbH & Co. Rheinische Straße,

Germany) through a 1 mm sieve. The dry matter (DM) content of the diets and fecal samples was measured in duplicate by method 930.15 (AOAC, 2007). Nitrogen content was determined in diet, fecal, and urine samples using an automatic analyzer (LECO FP 528; MI, USA; Method 990.03; AOAC, 2007). The gross energy (GE) content of the diets was analyzed by bomb calorimeter (6400 automatic Isoperibol system, Parr Instruments Company Illinois, USA). Total dietary fibre (**TDF**), soluble dietary fibre (**SDF**), and insoluble dietary fibre (**IDF**) of the complete diets were analyzed according to the AOAC (2007) method 991.43 using an ANKOM^{TDF} dietary fibre analyzer (ANKOM Technology, Macedon NY, USA). Acid-insoluble ash (**AIA**) content of the diets and fecal samples were measured in triplicates and duplicates, respectively, according to methods described by Van Keulen and Young (1997). Complete blood cell count was analyzed at Prairie Diagnostic Services (Saskatoon, SK, Canada) using an ADVIA 2120i haematology analyzer (Siemens Health Care Diagnostics, Deerfield, IL). Fibrinogen was analyzed by heat precipitate and refractometer method and serum albumin analyzed by bromocresol green method using a Cobas C 311 (Roche Diagnostics) according to Doumas et al. (1971). Serum haptoglobin was analyzed in the Animal Health Laboratory (University of Guelph, Guelph, ON) according to a method described by Makimura and Suzuki (1982) on a Roche Cobas 6000 c501 analyser.

4.3.5 Calculations

Apparent total tract digestibility of DM and N were determined using the indicator method according to the following equation:

$$N \text{ digestibility} = 100 - \left[\frac{ID \times NF}{IF \times ND} \right] \times 100\%$$

Where I_D and I_F is the indicator concentration (celite) in the diet and feces, respectively. N_D and N_F are the nitrogen concentration in the diet and feces, respectively. Nitrogen retention (g/d) was calculated as the difference between total N intake (g/d) and total N losses (urine and fecal N output; g/d). Whole-body protein deposition (g/d) was calculated as N retained \times 6.25.

4.3.6 Statistical Analyses

Statistical analyses were conducted using the PROC MIXED model procedure of SAS (version 9.4; SAS Institute, Inc. Cary, NC). Normality of the data was verified, and outliers were tested using the studentized residuals (PROC UNIVARIATE; SAS 9.4; SAS Institute, Inc. Cary, NC). Data for blood parameters were analyzed with dietary threonine ($n = 5$; fixed effect), fibre level ($n = 2$; fixed effect), period ($n = 2$; fixed effect) and block ($n=9$) as a random effect and interactions included in the model. For the N-balance data, analysis was completed separately for each period with fibre level ($n = 2$; fixed effect), Thr level ($n = 5$; fixed effect), and block ($n = 9$; random effect), as well as the interactions between fibre and Thr, included in the model as sources of variation. Orthogonal polynomial contrast statements were defined for each N-balance period to determine the linear and quadratic effects of dietary Thr concentration on PD. The quadratic break-point model was used (PROC NLIN) to estimate Thr requirement. The Tukey-Kramer mean separation test was used to determine differences between means and the significance level was defined as $P < 0.05$. A trend towards significance was considered at $P \leq 0.10$.

4.4 Results and Discussion

4.4.1 General Observation

Pigs were generally healthy throughout the experiment and consumed their daily feed allocations readily during the pre-ISS period. During the ISS period, the initial LPS injection generated a significant clinical response in pigs including signs of lethargy, feed refusal for some hours, and vomiting. Vomitus collected for each pig was included in the feed refusal for that day. The second injection did not result in any further observable clinical signs indicating the development of tolerance to the LPS (Rakhshandeh and de Lange, 2012). Five pigs were removed [LF (0.49, 0.73 and 0.57% SID Thr levels) and HF (0.73 and 0.49% SID Thr levels)] from the experiment due to incomplete urine and/or fecal collection. Analyzed and calculated nutrient content of experimental diets is presented in Table 4.2. Total dietary fibre content was higher (18.5%) in the HF diets than LF (12.5%) diets (Table 4.2). The total indispensable analyzed, and calculated AA contents of the experimental diets were similar across diets. The minor differences

are likely due to inherent inaccuracies in ingredient and diet sampling, diet preparation, and AA analysis (Rutherford and Moughan, 2000).

4.4.2 Response to LPS Challenge

In the present study, repeated intramuscular injection of LPS was selected as a non-infectious immune challenge model as previously reported (de Ridder et al., 2012; Litvak et al., 2013a). Taken together, the marked clinical observations, increased ($P < 0.01$) rectal temperature (Fig 4.1) and decreased WBC counts ($P < 0.001$) and major serum proteins ($P < 0.05$) measured after LPS injection (Table 4.3), all indicate effective stimulation of an acute inflammatory response. Acute leukopenia ($P < 0.001$) and a marked reduction ($P < 0.001$) in peripheral platelet count probably resulted from acute extravasation into tissues as has been observed by others following LPS injection in the pig (Kluess et al. 2015; Kvidera et al., 2017). Similarly, a reduction ($P < 0.001$) in serum albumin levels as reported in this study has also been reported by others following LPS in pigs (de Ridder et al., 2012; Litvak et al., 2013a; Christoffersen et al., 2015). Haptoglobin concentration decreased ($P < 0.05$) within 4 h of intramuscular LPS injection in the present study, which was surprising although possibly also related to acute capillary extravasation and the relatively short period between LPS administration and blood sampling. Others have reported an increase (Christoffersen et al. 2015) or no change (Gabay and Kushner, 1999) in haptoglobin levels following LPS administration. Reports suggest that the concentration of these acute-phase proteins increase 8 h after ISS and may remain elevated for about 24 h (Heegard et al., 1998; Hulten et al., 2003; Gruys et al., 2005). Increased ($P < 0.05$) fibrinogen concentration during the ISS period in the present study agrees with other studies (de Ridder et al., 2012; Litvak et al., 2013a) although the fibrinogen response to LPS observed here was variable (Table 4.3). To compensate for the expected development of tolerance to LPS (Rakhshandeh and de Lange, 2012), the second dose of LPS was increased by 15% (Rudar et al., 2016). Despite this increase in dose, no obvious clinical signs were observed following the second dose suggesting the increase in dose did not fully compensate for tolerance

Table 4.2 Analyzed nutrient composition of the experimental diets (as-is basis)

<i>Item</i>	SID ¹ Threonine, %									
	Low Fibre					High Fibre				
	0.49	0.57	0.65	0.73	0.81	0.49	0.57	0.65	0.73	0.81
GE, MJ/kg	16.60	16.49	16.48	16.59	16.66	17.16	17.14	17.34	17.25	17.20
DM, %	88.50	88.14	89.72	88.29	88.35	88.77	88.57	88.96	88.79	88.44
CP, %	19.69	18.33	19.14	19.46	18.71	18.73	18.62	19.29	18.97	18.85
TDF ² , %	12.47	12.25	12.00	12.86	12.54	17.80	18.21	17.95	18.72	18.93
SDF ³ , %	1.16	0.95	0.68	0.73	0.99	1.97	2.12	2.35	2.87	2.77
IDF ⁴ , %	11.31	11.30	11.25	12.13	11.54	14.50	16.09	15.60	15.84	16.16
Total Amino Acids⁵, %										
Lys	1.23(1.21)	1.25(1.21)	1.25(1.21)	1.25(1.21)	1.23(1.21)	1.20(1.22)	1.20(1.22)	1.25(1.22)	1.25(1.22)	1.28(1.22)
Met	0.42(0.45)	0.40(0.45)	0.45(0.45)	0.44(0.45)	0.44(0.45)	0.45(0.44)	0.45(0.44)	0.46(0.44)	0.45(0.44)	0.44(0.44)
Met+Cys	0.74(0.75)	0.72(0.75)	0.76(0.75)	0.76(0.75)	0.76(0.75)	0.76(0.76)	0.76(0.76)	0.78(0.76)	0.76(0.76)	0.74(0.76)
Thr	0.64(0.60)	0.67(0.66)	0.74(0.74)	0.80(0.82)	0.86(0.90)	0.64(0.60)	0.67(0.67)	0.75(0.75)	0.82(0.83)	0.89(0.90)
Trp	0.26(0.25)	0.25(0.25)	0.26(0.25)	0.27(0.25)	0.26(0.25)	0.25(0.25)	0.26(0.25)	0.27(0.25)	0.27(0.25)	0.26(0.25)
Arg	1.09(1.02)	1.02(1.02)	1.07(1.02)	1.06(1.02)	1.04(1.02)	1.04(1.03)	1.04(1.03)	1.07(1.03)	1.07(1.03)	1.07(1.03)
Ile	0.70(0.66)	0.68(0.66)	0.70(0.66)	0.70(0.66)	0.69(0.66)	0.68(0.67)	0.68(0.67)	0.69(0.67)	0.70(0.67)	0.70(0.67)
Leu	1.36(1.26)	1.30(1.26)	1.34(1.26)	1.34(1.26)	1.31(1.26)	1.29(1.27)	1.30(1.27)	1.33(1.27)	1.33(1.27)	1.32(1.27)
Val	0.89(0.82)	0.84(0.82)	0.87(0.82)	0.88(0.82)	0.86(0.82)	0.88(0.85)	0.87(0.85)	0.89(0.85)	0.90(0.85)	0.90(0.85)
His	0.44(0.41)	0.42(0.41)	0.44(0.41)	0.44(0.41)	0.43(0.41)	0.43(0.42)	0.43(0.42)	0.44(0.42)	0.44(0.42)	0.44(0.42)
Phe	0.88(0.79)	0.84(0.79)	0.87(0.79)	0.86(0.79)	0.85(0.79)	0.83(0.79)	0.84(0.79)	0.85(0.79)	0.85(0.79)	0.85(0.79)

¹SID = standardized ileal digestible.²TDF = total dietary fibre.³SDF = soluble dietary fibre.⁴IDF = insoluble dietary fibre.⁵Analyzed values of total amino acids with calculated values in brackets.

Table 4.3 Plasma concentration of selected proteins and white blood cell (WBC) count^{1, 2}

SID ³ Threonine, %															
Items	0.49		0.57		0.65		0.73		0.81		SEM	P-value ⁶			
	LF ⁴	HF ⁵	LF	HF	LF	HF	LF	HF	LF	HF		Period	Fibre	Thr	
WBC, ×10 ⁹ g/L															
Pre-ISS	24.8	20.5	20.5	19.9	20.6	20.9	22.9	22.3	25.8	20.1	1.40	<0.01	0.09	0.48	
ISS	4.5	4.8	4.4	4.9	4.3	5.1	5.2	3.9	4.6	4.9					
Platelets, ×10 ⁹ /L															
Pre-ISS	402.7	322.2	412.7	438.1	372.8	368.4	386.0	373.6	348.9	347.6	40.9	<0.01	0.56	0.08	
ISS	213.9	204.5	259.8	259.2	185.4	229.4	313.2	232.6	190.6	204.4					
Albumin, g/L															
Pre-ISS	38.8	36.0	37.0	37.0	38.3	36.4	40.7	38.2	39.7	39.1	1.10	<0.01	<0.05	<0.05	
ISS	35.9	32.5	34.3	34.3	34.2	32.8	37.5	34.7	34.9	36.7					
Haptoglobin, g/L															
Pre-ISS	0.64	0.66	0.49	0.54	0.64	0.85	0.78	0.54	0.72	0.58	0.17	<0.05	0.99	0.75	
ISS	0.60	0.34	0.39	0.58	0.38	0.54	0.65	0.56	0.34	0.46					
Fibrinogen, g/L															
Pre-ISS	2.7	1.3	2.1	1.5	2.4	1.8	2.6	2.1	1.3	1.2	0.32	<0.05	<0.05	<0.01	
ISS	2.3	1.4	3.9	2.6	2.9	1.8	3.1	1.8	2.3	2.4					

¹Immune system stimulation (ISS) was achieved by injecting increasing doses of *E. coli* lipopolysaccharide on day 1 of ISS at 30 µg/kg BW and 48hours after with a 15% increase from initial dose.

²The data presented are least-square means with a standard error of means (SEM) and based on blood samples taken before ISS and 4 h after ISS initiation (n=9).

³SID = Standard ileal digestible.

⁴LF = low fibre diet.

⁵HF= high fibre diet.

⁶No significant interactions were observed.

Table 4.4 Nitrogen (N) balance for low fibre (LF) high fibre (HF) diets during the pre-immune stimulation period¹

<i>Items</i>		SID²Threonine, %					SEM	<i>P-value</i>		
		0.49	0.57	0.65	0.73	0.81		<i>Fibre</i>	<i>Thr</i>	<i>Fibre × Thr</i>
Initial BW, kg	LF	24.1	25.2	24.8	25.2	24.9	0.40	0.49	0.19	0.14
	HF	24.9	24.8	25.4	24.5	25.3				
N Intake, g/d	LF	25.9	28.6	27.5	28.3	26.9	0.25	<0.05	<0.01	<0.01
	HF	26.8	26.3	27.7	27.1	27.1				
Fecal N output, g/d	LF	3.5	4.3	3.5	3.9	3.8	0.17	<0.01	0.14	<0.05
	HF	4.2	3.9	4.1	4.3	4.4				
Urinary N output, g/d	LF	3.2	3.3	2.5	2.1	1.9	0.35	<0.05	<0.01	0.48
	HF	3.1	2.2	2.6	1.5	1.4				
ATTD ³ N, %	LF	84.9	86.5	87.3	86.3	86.1	0.61	<0.01	0.11	0.10
	HF	84.1	85.2	85.2	84.1	83.7				
N retained, g/d	LF	19.2	21.02	21.51	22.36	21.28	0.45	0.15	<0.01	<0.05
	HF	19.5	20.25	21.02	21.27	21.33				
PD ⁴ , g/d	LF	120.1	131.4	134.4	139.7	133.1	2.81	0.15	<0.01	<0.05
	HF	121.6	126.6	131.4	132.9	133.3				

¹Data presented are least-square means with a standard error of means (SEM) and represent measurements taken before immune system stimulation (n=8-9).

²SID =Standard ileal digestible.

³ATTD= Apparent total tract digestibility.

⁴PD = protein deposition (N retained × 6.25).

Table 4.5 Nitrogen (N) balance for low fibre (LF) and high fibre (HF) diets during the immune stimulation period¹

<i>Items</i>		SID² Threonine, %					SEM	<i>P-value</i>		
		0.49	0.57	0.65	0.73	0.81		<i>Fibre</i>	<i>Thr</i>	<i>Fibre × Thr</i>
Initial BW, kg	LF	25.2	26.7	25.9	26.2	26.2	0.40	0.20	0.15	0.13
	HF	26.1	26.1	26.7	25.8	26.6				
N Intake, g/d	LF	24.8	27.1	25.9	27.2	25.6	0.58	0.24	0.06	0.05
	HF	25.8	25.7	27.1	26.9	27.3				
Fecal N output, g/d	LF	4.1	3.8	3.7	3.8	3.6	0.25	<0.05	0.99	0.34
	HF	4.2	3.9	4.3	4.2	4.4				
Urinary N output, g/d	LF	3.3	2.7	2.9	2.4	2.2	0.40	0.42	0.28	0.18
	HF	3.6	2.4	2.9	2.9	2.5				
ATTD ³ N, %	LF	84.7	84.9	85.5	85.9	86.1	0.82	<0.05	0.89	0.72
	HF	83.9	84.7	84.2	84.5	83.9				
N retained, g/d	LF	18.3	19.6	19.3	21.1	19.8	0.63	0.71	<0.05	0.10
	HF	17.9	19.3	19.9	19.9	20.4				
PD ⁴ , g/d	LF	114.4	122.7	120.9	131.4	123.9	3.95	0.73	<0.05	0.54
	HF	112.4	120.7	124.2	124.1	127.7				

¹Data presented are least-square means with a standard error of means (SEM) and represent measurements taken during immune system stimulation (n=8-9).

²SID =Standard ileal digestible.

³ATTD= Apparent total tract digestibility.

⁴PD = protein deposition (N retained × 6.25).

Table 4.6 Linear and quadratic relationship of dietary threonine and fibre on protein deposition (PD, g/d)

		SID ¹ Threonine, %					<i>P-value</i>		
		0.49	0.57	0.65	0.72	0.81	SEM	Linear	Quad ⁵
Pre-ISS ²									
	LF ³	119.7	131.5	134.4	140.0	133.2	2.88	<0.01	<0.01
	HF ⁴	121.3	126.6	131.4	133.3	133.3	3.04	<0.01	0.24
ISS									
	LF	114.4	123.2	120.7	131.1	124.9	4.12	<0.05	0.27
	HF	112.5	120.7	124.2	123.9	127.3	3.80	<0.05	0.36

¹SID = standardized ileal digestible.²ISS= Immune system stimulation.³LF = Low fibre diet.⁴HF = High fibre diet.⁵Quad; Quadratic contrasts

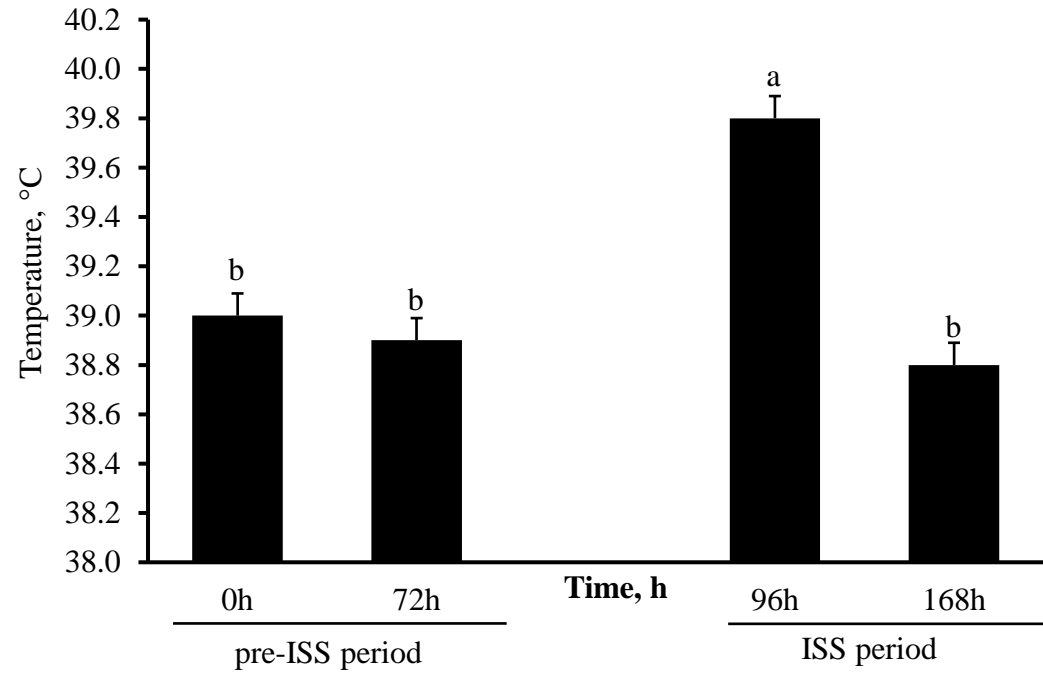
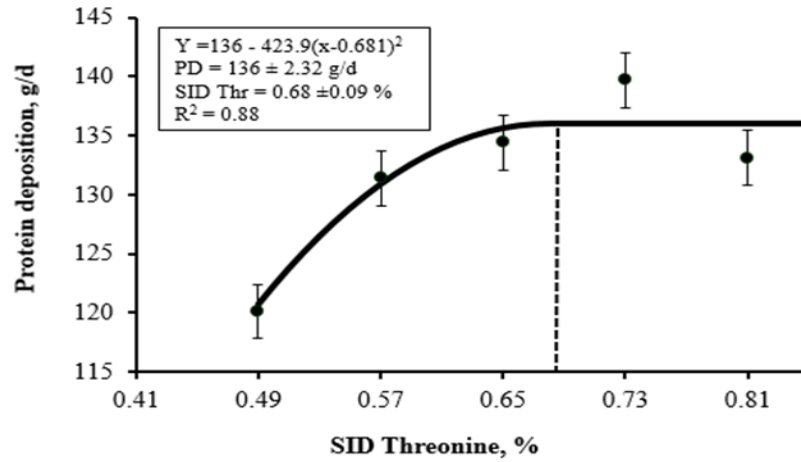


Figure 4.1 Rectal temperature measured at time 0 and 72 h (pre-immune system stimulation period) and 96 and 168 h (immune system stimulation period). LPS administered intramuscularly at 92 h and 140 h with an observed significant difference between rectal temperatures taken at the various points ($P < 0.01$).

A



B

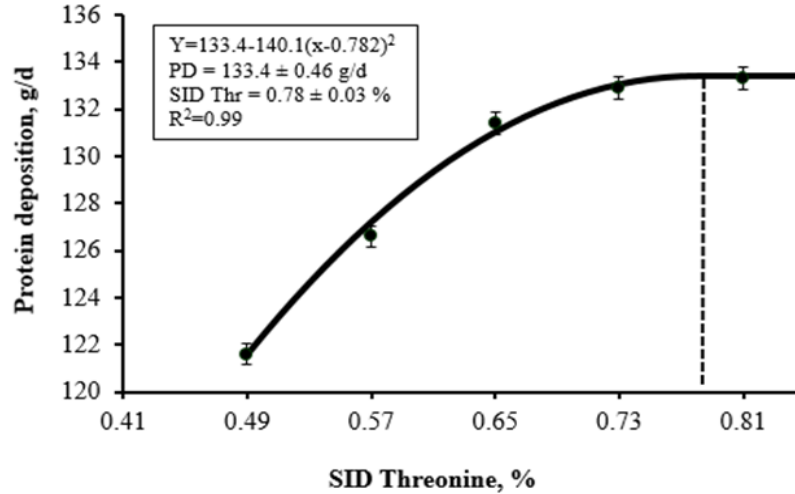


Figure 4.2 The quadratic break-point model analysis estimates during the pre-immune system stimulation period for low fibre (**A**) and high fibre (**B**). Low fibre diets show a breakpoint at 0.68% SID Thr for maximum protein deposition (PD) at 136 g/d. High fibre diets show a breakpoint at 0.78% SID Thr for maximum PD at 133 g/d.

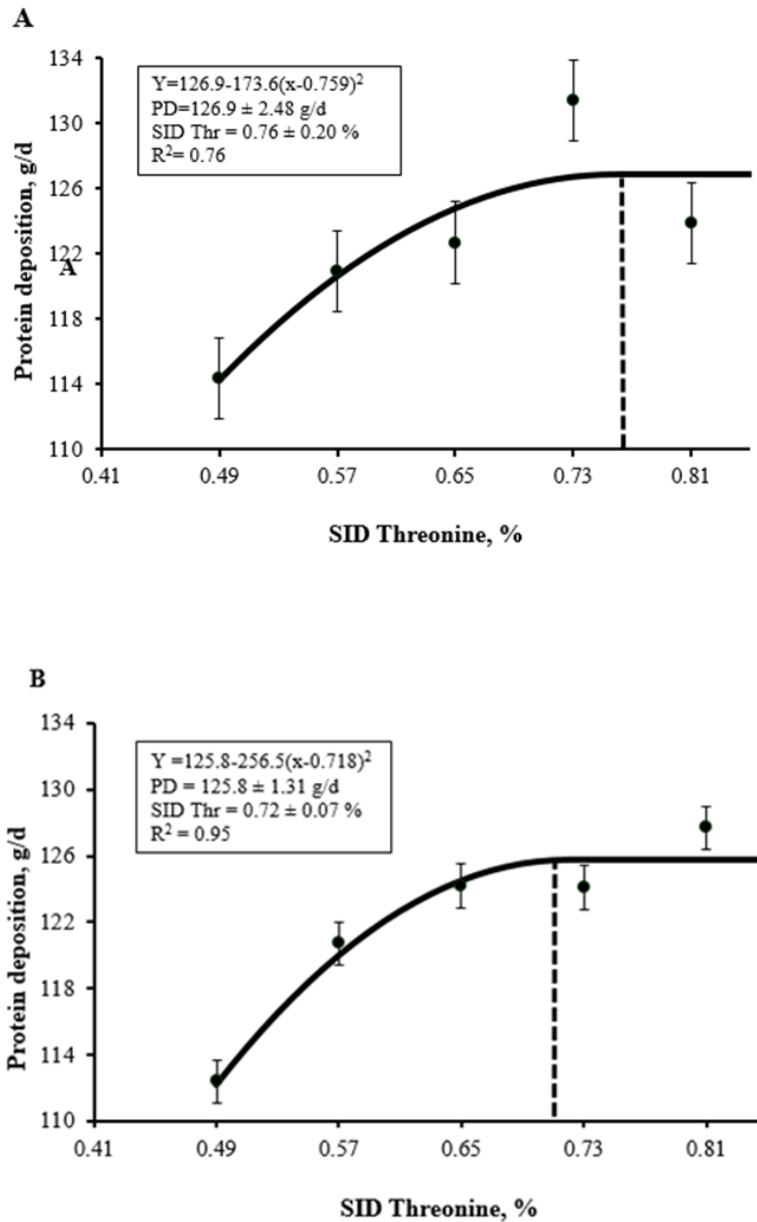


Figure 4.3 The quadratic break-point model analysis estimates during the immune system stimulation period for LF (**A**) and high fibre (**B**). Low fibre diets show a breakpoint at 0.76% SID Thr for maximum protein deposition (PD) at 127 g/d. High fibre diets show a breakpoint at 0.72% SID Thr for maximum PD at 126 g/d.

4.4.3 Nitrogen Balance in Response to Dietary Fibre and Threonine Dose

The use of co-products has been increasing in swine production, and these products tend to increase the fibre content in swine diets. Fibrous ingredients will increase digesta passage rate and microbial activity in the hindgut (Lindberg, 2014). According to Stein et al. (1999), Thr is highly present in endogenous AA losses, and Zhu et al. (2005) therefore suggested that increasing fibre contents in swine diets will increase the requirement for Thr in growing pigs. Mathai et al. (2016) have shown that when DF content is increased, Thr:Lys ratio increased for optimal growth in pigs fed a corn-soybean meal diet. Therefore, the present study evaluated individually the effects of high DF and ISS on PD and these effects were also combined to test their additivity on PD.

When no ISS was present, N intake showed a significant interaction ($P < 0.05$; Table 4.4) between fibre level and Thr concentration, with total N intake increasing as Thr intake increased in both LF and HF diets ($P < 0.01$). High DF increased ($P < 0.01$) fecal N output, an observation consistent with other studies and representing a shift in N excretion from urine to feces (Dilger et al., 2004; Libao-Mercado et al., 2006). This is confirmed by the decrease in urine N output in HF-fed pigs compared with pigs fed LF diets ($P < 0.05$). Regardless of the DF level, as Thr concentration in the diet increased, N output in urine decreased, an indication of increased N utilization. Apparent total tract digestibility of N was lower ($P < 0.01$) in pigs fed the HF diet than in pigs fed the LF diets, which confirms earlier reports on the negative effect of fibre on N digestibility (Bach Knudsen and Hansen, 1991; Stein et al., 1999; Bach Knudsen et al., 2012). There was a significant interaction ($P < 0.05$) between DF and dietary Thr on PD. Generally, as dietary Thr increased, PD increased in both HF and LF diets, however, the rate of increase was reduced in pigs fed HF diets compared to pigs fed LF diets and suggests that requirement for Thr may be greater in HF than in LF diets. This confirms earlier reports (Myrie et al., 2008; Mathai et al., 2016) suggesting that high DF reduces N retention and PD. This has been shown to be due to fibre associated Thr losses, which depends on the fibre source and the concentration in the diet (Blank et al., 2012). In the present study, wheat bran and sugar beet pulp were used as a source of insoluble and soluble fibre, respectively, which have been reported to contribute to endogenous Thr losses due to an increase in mucosal protein synthesis, particularly mucins which are rich in Thr (Zhu et al., 2005; Libao-Mercado et al., 2006, 2007; Blank et al., 2012).

During the ISS period, N intake and urinary N output were not affected ($P > 0.05$; Table 4.5) by dietary Thr concentration or fibre level. Fecal N output; however, increased ($P < 0.05$) in pigs fed HF diets but was not affected by dietary Thr level ($P > 0.05$). This is comparable to observations made when no ISS was present. Nitrogen retained, and PD was affected ($P < 0.05$) by dietary Thr concentration, such that as dietary Thr increased, PD increased linearly. There was no significant effect of DF and no interaction observed. This linear increase in PD with increasing Thr suggests that to achieve maximum PD during ISS, higher dietary Thr will be required. Further, this is related to the systemic effect of LPS injection, which is expected to increase Thr requirement for PD due to the repartitioning of AA towards maintaining an immune response and prioritization of immune function to growth (Obled, 2003; Reeds et al., 1994; Munasinghe et al., 2017). Although statistically, we did not compare the pre-ISS period to the ISS period, N retained, and PD were numerically less in pigs fed LF diets when ISS was present compared to the values for pigs fed LF diets without ISS. This appears that ISS independently reduces N retention and PD. During ISS, high DF did not affect N retention and PD ($P > 0.05$; Table 4.5). Similarly, comparing numerically between high HF during pre-ISS with HF during ISS, PD was numerically decreased during the ISS period. However, we cannot exactly separate the singular effects of ISS and HF on PD but suggests that high DF and ISS do not act additively to further reduce N retention and PD.

4.4.4 Threonine Requirement for ISS and Fibre

Broken-line regression can be used to analyze nutrient dose-response data using either linear or quadratic functions to estimate a breakpoint which is interpreted as the nutrient requirement (Robbins et al., 2005). In the current study, dose-response data were analyzed using both linear and quadratic break-point model, however, the quadratic break-point model provided the best fit to the data according to the coefficient of determination (R^2) and so only results from this model are presented.

When no ISS was present, we observed an increase in the estimate of Thr requirement for PD in the HF-fed pigs compared to the LF-fed pigs. The Thr requirement for LF diets for maximum PD was estimated at 0.68% SID ($R^2 = 0.88$) and for HF diets at 0.78% SID ($R^2 = 0.99$) as shown in Fig. 4.2A and 4.2B, respectively. The estimated Thr requirement in LF diets was higher than

0.61% SID Thr suggested by NRC (2012) for pigs of 25-50 kg BW but lower than the value estimated by Mathai et al. (2016) at 0.72% SID Thr required to optimize growth performance in gilts between 25-50 kg fed LF diets. Similarly, Zhang et al. (2013b) estimated 0.64% SID Thr requirement in 22-50 kg when fed LF diets with low crude protein levels. The SID Thr required to maximize PD in a HF diet was estimated at 0.78% in the present study and suggests that high DF increases Thr requirement, which is consistent with Mathai et al. (2016) who estimated 0.77% SID Thr required to optimize growth performance in gilts fed a high DF. The NRC, (2012) acknowledges the effect of high DF on threonine requirement and therefore provides an adjustment in requirements when high fibre diets are fed to pigs. Others have reported a reduction in growth performance or PD and effects on Thr when feeding high fibre diets (Zhu et al., 2005; Libao-Mercado et al., 2006; Myrie et al., 2008).

During ISS period, Thr requirement for LF diets was estimated at 0.76% SID (Fig 4.3A), which was higher than the 0.68% SID Thr estimated when ISS was absent, this suggests that ISS increases the requirement for Thr to maximize PD. As far as we know, this is the first study to quantify the effect of ISS on Thr requirements in pigs for PD. Some studies have observed changes in immune status (Li et al., 1999) and growth performance of pigs when immune challenged (Zhang et al., 2013b) and graded levels of Thr are fed. Others have quantified requirement for Met to Met+Cys ratio (Litvak et al., 2013a), Leu requirements (Rudar et al., 2016), and utilization efficiency of tryptophan for PD (de Ridder et al., 2012). These studies used the LPS model to initiate ISS as used in the present study and have all reported that ISS increases the requirement for the AA under investigation. Litvak et al. (2013a) reported an increase in Met to Met+Cys ratio during an LPS challenge. Similarly, de Ridder et al. (2012) reported a reduction in Trp utilization efficiency for PD in growing pigs challenged with LPS. Therefore, the increase in Thr requirement estimated in the present study during ISS agrees with the previous reports. Of the AA, Glu, Arg, Thr, and aromatic and sulphur AA are of importance during immune challenge (Reeds and Jahoor, 2001). Threonine is especially important for the synthesis of many acute-phase proteins and immunoglobulins during an immune response (Reeds et al., 1994; Li et al. 2007) explaining the increase in Thr requirement for PD with ISS. Furthermore, Rakhshandeh et al. (2013) demonstrated increased intestinal MUC2 expression, abundant in Thr, following systemic LPS challenge in pigs. In the present study, we also evaluated the combined effects of feeding high DF to pigs when ISS was present on Thr requirement for PD. As shown in Fig. 4.3B, estimated Thr

requirement for PD was lower (0.72% SID Thr) compared to the estimates of 0.78% SID Thr required for PD with feeding high DF diets when ISS was absent, and 0.76% SID Thr required for PD with feeding LF diets during ISS. We hypothesized that feeding high DF diets with ISS would have an additive effect on the amount of Thr required to maximize PD; however, the results suggest a non-additive effect. The mechanisms associated with this observation are not clear but could be related to the effect of high DF increasing mucous secretion mediated by a local physiological inflammatory response to the DF such that LPS injection did not contribute to any further inflammatory response in intestinal mucosa and hence the need for increased Thr requirement to support added mucous secretion. We further hypothesize that the injection of LPS induced some physiological inflammation in the intestine when pigs were fed the LF diets, but in case of the high DF diets, the intestine is refractory to the influence of systemic LPS challenge. It should also be noted that the plateau, and therefore the requirement, in HF-fed pigs during ISS is based on few data points and therefore could be higher.

4.5 Conclusion

In the present study, we have confirmed that high DF reduces N retention and PD efficiency and further quantified that SID Thr of 0.78% is required to maximize PD when feeding pigs high DF. We also quantify for the first time the effect of ISS on Thr requirement for PD, which translates to 0.76% SID Thr required to maximize PD in growing pigs from 25-50 kg. Further, the present study shows that combining the effects of high DF and ISS did not additively increase Thr required to maximize PD, suggesting that DF could mask the ISS effects on Thr required for maximum PD. The mechanisms explaining the lack of additive effects require clarification. Although there are some variations in these estimates as reported by other authors, these variations could be partly accounted for by the differences in the response parameters (e.g. PD, ADG) measured and the type and concentration of fibre used in these studies as well as the regression models used to analyze and interpret the data. However, this finding is important because of the increasing use of co-products in swine diets and the resulting increase in DF. Therefore, understanding changes and defining Thr requirement under the range of conditions experienced commercially will lead to improved nutritional programs that will increase production efficiency and profitability and reduce the environmental impact of pork production.

4.6 Acknowledgements

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CHAPTER 5

ESTIMATING THE OPTIMAL THREONINE REQUIREMENT FOR 25-50 KG PIGS FED A MIXTURE OF SOLUBLE AND INSOLUBLE DIETARY FIBRE

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Author Contributions

DAC, AVK and MOW conceived and designed the experiments. MOW carried out the experiment analyzed all data JKH assisted with data analysis and edited the manuscript. MOW drafted the manuscript. All authors read and approved the manuscript.

5.1 Abstract

In the current swine production systems, high amounts of co-products are used in diet formulation, this has increased the dietary fibre (**DF**) content in swine diets. High DF is reported as one of the factors that may contribute to increased threonine (**Thr**) requirement. Previous studies have demonstrated a reduced efficiency of utilization of dietary Thr for body PD when high-fibre feedstuffs are included in the diet of growing pigs. A growth performance experiment was conducted to estimate Thr requirement in 25-50 kg pigs fed diets containing a mixture of soluble and insoluble DF. A total of 160 growing pigs ($27.1 \pm \text{SD} = 0.6$ kg) housed 4pigs/pen (2 gilts and 2 barrows) and pens randomly assigned to 1 of 5 wheat soybean-based diets formulated to contain 10% sugar beet pulp and 5% wheat bran (17.5% total DF) with 5 graded Thr levels [0.66, 0.71, 0.76, 0.81 and 0.86% standardized ileal digestible (**SID**)]. All other AA were balanced to meet or exceed the requirement for 25-50 kg pigs using the analyzed ingredient AA contents and published SID coefficients. Data were analyzed by PROC MIXED (SAS, 9.4) as a randomized complete block design with the Thr level as a fixed effect and room (block) as a random effect. Orthogonal polynomial contrast statements were defined to determine linear and quadratic relationship between dietary Thr concentration and the defined response parameters in the present study. Optimal Thr requirement was estimated using PROC NLIN linear and quadratic break-point models. After a 26-d growth performance study, results indicate significant linear and the quadratic response of average daily gain (**ADG**) and gain to feed (G:F) as Thr increased with no significant effect on average daily feed intake (**ADFI**). Linear and quadratic broken line breakpoint models estimated 0.76% and 0.80% SID Thr to maximize ADG and 0.76 and 0.81% SID Thr to maximize G:F, respectively. In conclusion, this study demonstrated that high DF would increase Thr requirement for maximum growth performance, however, the source, type, and concentration of fibre will influence the rate of increase.

5.2 Introduction

The efficiency of protein utilization for growth is affected by the balance of essential AA in the protein as well as other dietary and physiological factors (Stein et al., 1999; Mathai et al., 2016). Threonine is usually the second limiting AA in practical swine diets which, if included at appropriate levels, will not only enhance growth performance but also contribute to maintaining immune status and intestinal barrier function (Li et al., 1999; Wang et al., 2006; 2010). However, large variations exist among the reported requirements for Thr in pigs of 25 to 50 kg body weight (BW), with reported values ranging between 0.60 to about 0.77% SID content of the diet (Blank et al., 2012; NRC, 2012; Zhang et al., 2013a; Mathai et al., 2016). Dietary fibre (**DF**) is reported as one of the factors that may contribute to increased Thr requirement. Previous research has demonstrated a reduced efficiency of utilization of dietary Thr for body PD when high-fibre feedstuffs (i.e., wheat shorts, pectin) are included in the diet of growing pigs (Zhu et al., 2005; Libao-Mercado et al., 2006). This effect was attributed to an increase in the endogenous Thr losses due to increase production of mucin, a Thr rich glycoprotein (Libao-Mercado et al., 2007). In current swine production systems, there is an increased focus on supporting animal health and increased use of co-products in swine diet formulations which have resulted in increased DF content. Therefore, understanding the impact of DF on Thr requirement for growing pigs is of increasing practical importance. The current NRC, (2012) adjusts Thr requirements based on the level of soluble (fermentable) DF content. However, different fibre sources will contain different types of fibre (i.e., soluble and insoluble) and, as reported by Bach-Knudsen and Hansen (1991), different fibre types will have different physicochemical properties with differential effects on digestive physiology. The objective of the current study was to quantify Thr requirement in 25-50 kg pigs fed both soluble and insoluble fibre sources by ADG) and G:F as response variables.

5.3 Materials and Methods

The experimental protocol was reviewed and approved by the University of Saskatchewan's Animal Research Ethics Board (protocol #20130054) and followed the Canadian Council on Animal Care guidelines (CCAC, 2009).

5.3.1 Animal, Diet, and Experimental Design

A total of 160 growing pigs (Camborough Plus \times C3378; PIC Canada Ltd.) with an initial BW of $27.1 \pm \text{SD}=0.6$ kg was used in a growth performance study at the Prairie Swine Centre, Inc. (Saskatoon, SK, Canada). Pigs were randomly grouped into slatted floor pens of 4 pigs/pen (2 gilts and 2 barrows). The experimental design was a randomized complete block design, and pens were assigned ($n=8$) to 1 of 5 dietary treatments. The diets were wheat and barley-based (Table 5.1) and formulated to contain 10% sugar beet pulp and 5% wheat bran as sources fibre and 1 of 5 levels of SID Thr (0.66, 0.71, 0.76, 0.81 and 0.86%). All other essential AA were balanced at 110% of NRC (2012) requirement for 25-50 kg pigs using the analyzed ingredient AA contents and published SID coefficients (NRC, 2012). Pigs were fed *ad libitum* and had unrestricted access to water for the duration of the study.

5.3.2 Experimental Procedure and Nutrient Analyses

Pigs were weighed on d 0 (start of the experiment) and on d 7, 14, 21 and 26 to calculate ADG. Similarly, feed offered, and feed weighed back were monitored weekly and used to calculate ADFI and G: F. Diet samples were analyzed for AA composition using ion-exchange chromatography with post-column derivatization with ninhydrin (AMINOLab of Evonik Nutrition & Care GmbH, Hanau, Germany; Llames and Fontaine, 1994). The dry matter (DM) content of the diets was measured in duplicate by method 930.15 (AOAC, 2007). Nitrogen (N) content of the experimental diets was determined in duplicates using an automatic analyzer (LECO FP 528; MI, USA; Method 990.03; AOAC, 2007) and the crude protein (CP) calculated as $\text{N} \times 6.25$. Total dietary fibre (**TDF**), soluble dietary fibre (**SDF**) and insoluble dietary fibre (**IDF**) of the complete diets (Table 5.2) were analyzed in duplicate according to the AOAC (2007) method 991.43 using an ANKOM^{TDF} dietary fibre analyzer (ANKOM Technology, Macedon NY, USA).

Table 5.1 Composition of the experimental diets (as-fed basis)

Ingredients, %	Standardized ileal digestible (SID) threonine, %¹				
	0.66	0.71	0.76	0.81	0.86
Corn	4.70	4.70	4.70	4.70	4.70
Wheat	48.00	48.00	48.00	48.00	48.00
Barley	7.00	7.00	7.00	7.00	7.00
Canola oil	3.70	3.60	3.60	3.50	3.50
Soybean meal	18.50	18.50	18.50	18.50	18.50
Wheat bran	5.00	5.00	5.00	5.00	5.00
Sugar beet pulp	10.00	10.00	10.00	10.00	10.00
L-Thr	0.19	0.23	0.28	0.33	0.37
L-Lys·HCl	0.56	0.56	0.56	0.56	0.56
DL-Met	0.20	0.20	0.20	0.20	0.20
L-Trp	0.03	0.03	0.03	0.03	0.03
L-Val	0.09	0.09	0.09	0.09	0.09
L-Leu	0.07	0.07	0.07	0.07	0.07
Limestone	1.00	1.00	1.00	1.00	1.00
Monocalcium phosphate	0.70	0.70	0.70	0.70	0.70
Salt	0.20	0.20	0.20	0.20	0.20
Vitamin-mineral premix ²	0.10	0.10	0.10	0.10	0.10
Calculated nutrient contents³					
DM, %	84.38	84.44	84.48	84.53	84.57
CP, %	17.86	17.9	17.93	17.97	18.00
ME, kcal/kg	3345	3339	3341	3334	3336
NE, kcal/kg	2506	2501	2502	2496	2497
SID Lys, %	1.12	1.12	1.12	1.12	1.12
SID Thr, %	0.66	0.71	0.76	0.81	0.86
SID Met+Cys, %	0.68	0.68	0.68	0.68	0.68
SID Trp, %	0.22	0.22	0.22	0.22	0.22
SID Val, %	0.75	0.75	0.75	0.75	0.75
SID Ile, %	0.59	0.59	0.59	0.59	0.59
SID Leu, %	1.12	1.12	1.12	1.12	1.12

¹The experimental diets with the intermediate % SID Thr (0.71, 0.76 and 0.81) were prepared by blending the 0.66 and 0.86 diets in appropriate proportions.

²Supplied per kg of complete diet: vitamin A, 8000 IU; vitamin D, 1500 IU; vitamin E, 30 IU; menadione, 2.5 mg; vitamin B12, 0.025 mg; thiamine, 1.00 mg; biotin, 0.10 mg; niacin, 20 mg; riboflavin, 4 mg; pantothenate, 12 mg; folic acid, 0.50 mg; pyridoxine, 2.0 mg; Fe, 100 mg; Zn, 100 mg; Mg, 40 mg; Cu, 15 mg; Se, 0.30 mg and I, 1mg.

³Nutrient contents of diets were estimated based on the analyzed ingredient AA contents and published SID coefficients (NRC, 2012).

Table 5.2 Analyzed nutrient compositions of the experimental diet (as-is basis)

	Standardized ileal digestible threonine, %				
	0.66	0.71	0.76	0.81	0.86
DM, %	89.42	89.48	89.53	89.41	89.48
CP, %	19.31	19.38	19.12	19.06	19.25
SDF ¹ , %	2.7	3.2	2.5	2.5	2.7
IDF ² , %	15.0	14.6	14.6	15.0	14.9
TDF ³ , %	17.7	17.8	17.1	17.5	17.6
Total AA⁴, %					
Lys	1.20(1.22)	1.20(1.22)	1.25(1.22)	1.25(1.22)	1.28(1.22)
Met	0.45(0.44)	0.45(0.44)	0.46(0.44)	0.45(0.44)	0.44(0.44)
Met+Cys	0.76(0.76)	0.76(0.76)	0.78(0.76)	0.76(0.76)	0.74(0.76)
Thr	0.82(0.78)	0.84 (0.82)	0.88 (0.87)	0.89 (0.92)	0.96 (0.96)
Trp	0.25(0.25)	0.26(0.25)	0.27(0.25)	0.27(0.25)	0.26(0.25)
Arg	1.04(1.03)	1.04(1.03)	1.07(1.03)	1.07(1.03)	1.07(1.03)
Ile	0.68(0.67)	0.68(0.67)	0.69(0.67)	0.70(0.67)	0.70(0.67)
Leu	1.29(1.27)	1.30(1.27)	1.33(1.27)	1.33(1.27)	1.32(1.27)
Val	0.88(0.85)	0.87(0.85)	0.89(0.85)	0.90(0.85)	0.90(0.85)
His	0.43(0.42)	0.43(0.42)	0.44(0.42)	0.44(0.42)	0.44(0.42)
Phe	0.83(0.79)	0.84(0.79)	0.85(0.79)	0.85(0.79)	0.85(0.79)

¹Soluble dietary fibre.²Insoluble dietary fibre.³Total dietary fibre.⁴Analyzed total amino acids (AA) with calculated values in parenthesis.

5.3.3 Statistical Analyses

All data were checked for normality and outliers were verified using PROC UNIVARIATE in SAS (SAS Inst. Inc., Cary, NC). Data were analyzed by ANOVA using PROC MIXED in SAS as a randomized complete block design (SAS Inst. Inc., Cary, NC) with a pen as the experimental unit ($n=8$). The model included dietary Thr level as a fixed effect ($n=5$) and block (room) as a random effect ($n=2$). Orthogonal polynomial contrasts were used to determine the linear and quadratic effects of increasing dietary Thr on ADG and G:F. Significant differences were declared at $P \leq 0.05$ using LSMEANS statements and mean separation by least significant difference (LSD) method (PDIFF option in SAS 9.4 Inst. Inc., Cary, NC). The overall treatment means for ADG and G:F was analyzed using PROC NLIN in SAS to determine linear broken-line and quadratic broken-line model estimates for optimal dietary Thr requirement. The broken line analysis was fitted as $[y = L + U \times (R-x)]$, where $(R-x)$ was defined as zero at values of $x > R$; Robbins et al., 2006]. The quadratic broken-line was fitted as $[y = L + U \times (R-x) \times (R-x)]$, where $(R-x)$ is zero at values of $x > R$ and the plateau L at values $x > R$; Robbins et al., 2006].

5.4 Results

5.4.1 Growth Performance

Growth performance data are presented in Table 5.3. Increasing the dietary SID Thr concentration did not affect the overall ADFI (linear, $P = 0.766$; quadratic $P = 0.635$), however, during week 4 (d 22-26) there was a linear increase in ADFI (linear, $P < 0.01$). The overall ADG reported in this study showed both linear and quadratic responses as dietary Thr level increased (linear, $P < 0.01$; quadratic, $P < 0.01$). There were no significant differences in the initial BW among dietary treatments (linear, $P = 0.97$; quadratic, $P = 0.56$), however, as dietary SID Thr concentration increased, the final BW increased (linear, $P < 0.01$; quadratic, $P < 0.01$). Increasing dietary SID Thr increased the overall G:F (linear, $P < 0.01$, quadratic, $P < 0.01$).

5.4.2 Response to Graded Dietary Threonine

The linear and quadratic broken line models were used to estimate the SID Thr concentration required to optimize ADG and G:F. Dietary SID Thr required to maximize ADG was estimated at 0.76% (Fig 5.1A) and 0.80% (Fig 5.1B) using linear broken-line and quadratic broken-line models, respectively. For G:F, 0.76% (Fig 5.2A) and 0.81% (Fig 5.2B) SID Thr was required to optimize G:F according to linear broken-line and quadratic broken-line model, respectively.

Table 5.3 Increasing standardized ileal digestible threonine levels on growth performance of pigs fed high fibre diets¹

<i>Item</i>	Standardized ileal digestible (SID) Thr, %					Contrasts (<i>P</i> -value)		
	0.66	0.71	0.76	0.81	0.86	SEM ²	Linear ³	Quad ⁴
Body Weight, kg								
Initial	27.0	27.1	27.3	27.1	27.0	0.54	0.97	0.56
Final	49.3	50.4	52.6	52.0	51.8	0.59	<0.01	<0.01
Average daily feed intake, g								
Day 0-7	1372	1353	1356	1324	1355	34.9	0.21	0.27
Day 8-14	1736	1713	1715	1725	1736	18.9	0.90	0.39
Day 15-21	1861	1876	1849	1841	1844	45.1	0.48	0.92
Day 22-26	1911	1980	2009	1911	2037	22.6	<0.01	0.84
Overall	1721	1730	1752	1702	1741	14.3	0.77	0.64
Average daily gain, g								
Day 0-7	944	910	866	811	840	39.5	<0.01	0.35
Day 8-14	828	898	978	1000	1008	20.3	<0.01	0.02
Day 15-21	884	929	995	1007	972	18.4	<0.01	<0.01
Day 22-26	787	841	1056	1004	987	28.1	<0.01	<0.01
Overall	860	894	974	955	952	13.1	<0.01	<0.01
Gain:feed								
Day 0-7	0.69	0.67	0.64	0.61	0.62	0.023	0.01	0.55
Day 8-14	0.48	0.52	0.57	0.58	0.58	0.013	<0.01	<0.05
Day 15-21	0.47	0.05	0.54	0.55	0.53	0.016	<0.01	0.03
Day 22-26	0.41	0.42	0.53	0.53	0.48	0.015	<0.01	<0.01
Overall	0.50	0.52	0.56	0.56	0.55	0.008	<0.01	<0.01

¹Data presented are means with $n=8$ (pens) per treatment.²Standard error of mean³Linear response.⁴Quadratic response

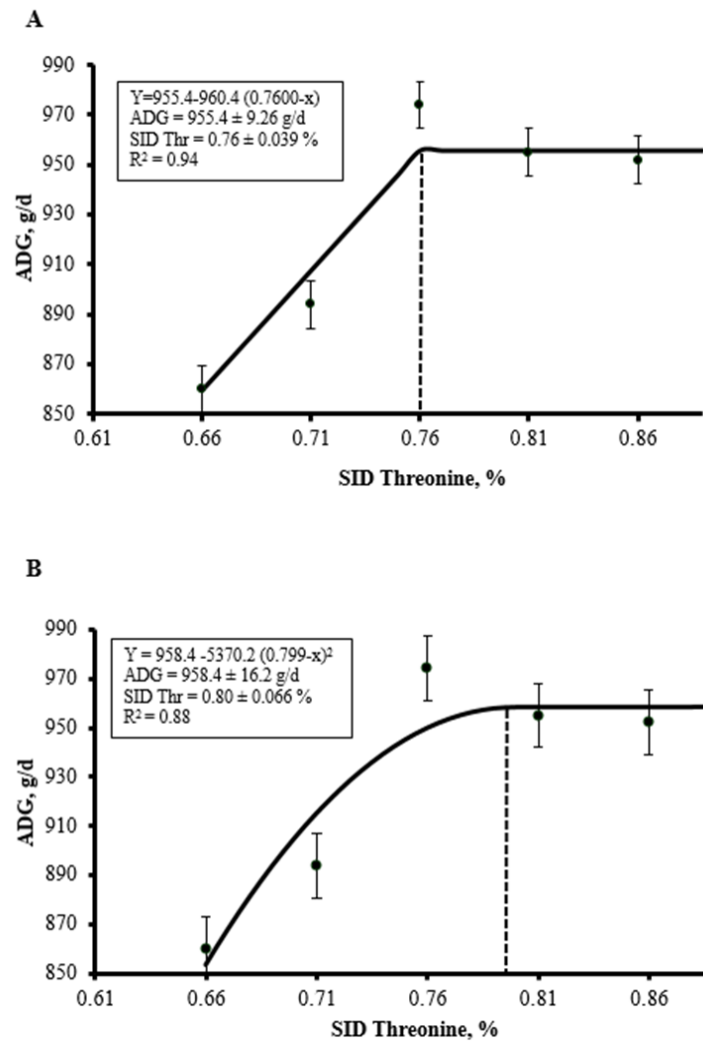


Figure 5.1 Broken line plot of ADG as a function of standardized ileal digestible threonine. Linear broken line (A) estimates a breakpoint at 0.76% SID Thr to maximize ADG at 955.4 g/d. Quadratic broken line (B) estimates a breakpoint at 0.80% SID Thr to maximize ADG at 958.4 g/d.

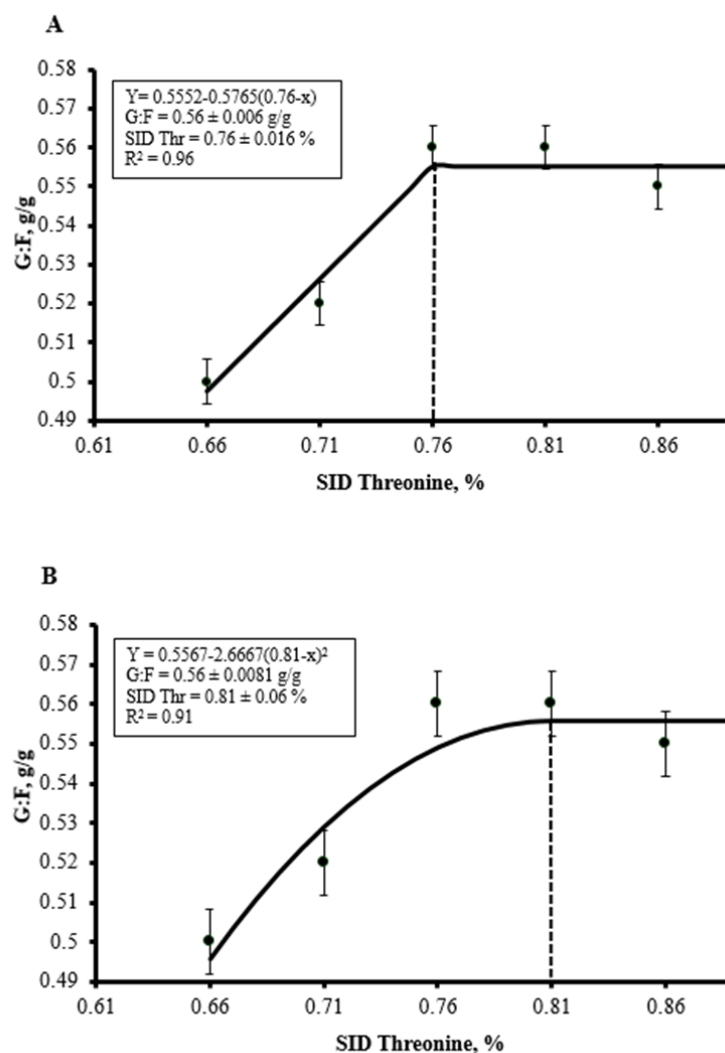


Figure 5.2 Broken line plot of Gain:feed (G:F) as a function of standardized ileal digestible threonine. Linear broken line (A) estimates a breakpoint at 0.76% SID Thr to maximize G:F at 0.56 g/g. Quadratic broken line (B) estimates a breakpoint at 0.81% SID Thr to maximize G:F at 0.56 g/g.

5.5 Discussion

The aim of the present study was to determine the SID Thr level required to maximize ADG and G:F in 25-50 kg growing pigs fed diets high in DF. Earlier reports have suggested that high DF will influence Thr requirement for optimal growth, however, these studies did not quantify the increase (Zhu et al., 2005; Libao-Mercado et al., 2006; Blank et al., 2012). Recently, Mathai et al. (2016) quantified Thr requirement when feeding high fibre diets to gilts of 25-50 kg. The results suggested 0.77% SID Thr required to maximize ADG using soybean hulls as the high fibre source. In the current study, we present data from pigs fed diets containing 10% wheat bran and 5% sugar beet pulp as sources of fibre which corresponds to 17.5% TDF. The data suggest that 0.78% SID Thr was required to maximize ADG which is 32% higher than the NRC (2012) recommendation of 0.59% SID Thr for 25-50 kg pigs. The estimated Thr requirement in the present study agrees with 0.77% SID Thr reported by Mathai et al. (2016) when growing pigs 25-50 kg were fed diets containing a 15% soybean hulls. Blank et al. (2012) assessed endogenous Thr losses using different fibre sources and concluded that Thr losses per gram of DF depend on the fibre source and the concentration. This suggests that fibre source, type (soluble vs. insoluble) and concentration in the diet may all influence Thr requirement. The NRC (2012) therefore considers the effect of fibre on Thr requirement and recommends an adjustment to dietary Thr levels when fermentable fibre level is high in the diet, however, this adjustment only account for soluble fibre content and therefore should be refined based on the variations in Thr requirement observed here and by others using a variety of fibre sources, including insoluble and mixed source fibre.

5.6 Conclusion

Threonine required to maximize both ADG and G:F is increased for 25-50 kg growing pigs when fed diets containing high DF included as wheat bran and sugar beet pulp. The results of this study support earlier reports that high DF increases Thr requirements. However, given the variability in source and concentration of fibre used in the current and previous studies, further research is required to more fully understand and quantify the effects of different fibre sources.

5.7 Acknowledgements

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CHAPTER 6
EFFECT OF SUPPLEMENTAL THREONINE ABOVE REQUIREMENT ON GROWTH
PERFORMANCE OF *SALMONELLA TYPHIMURIUM* CHALLENGED PIGS FED
HIGH FIBRE DIETS

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Author Contributions

DAC, AVK, MOW and JKH conceived and designed the experiment. MOW, AKA, KH carried out the experiment. MOW analyzed all data and drafted the manuscript. All authors read and approved the manuscript.

6.1 Abstract

It was shown previously that high dietary fibre (**DF**) and immune system stimulation (**ISS**) with systemic *E. coli* lipopolysaccharide (**LPS**) independently increased the threonine (**Thr**) requirement to maximize growth performance and protein deposition (**PD**). However, no additive effects on the Thr requirement were observed when both DF and ISS were present. The objective of the present study was to investigate whether supplementing Thr to meet high DF and systemic immune challenge requirements would maintain the performance of pigs exposed to an enteric immune challenge when fed high DF. A total of 128 pigs ($22.6 \pm \text{SD}=1.6$ kg initial BW) were assigned to 1 of 4 dietary treatments in a 2×2 factorial arrangement in a randomized complete block design ($n=8$ pens/treatment and 4 pigs/pen) for 28-d. Treatments were a low fibre (**LF**; 13% **TDF**) or high fibre (**HF**; 20% TDF) diet with either a standard (STD; 0.65% SID) or supplemental (SUP; 0.78% SID) Thr level. After a 7-d adaptation, pigs were orally inoculated with 2 mL (2.3×10^9 CFU/mL) of *Salmonella typhimurium* (**ST**). Blood samples and rectal swabs were obtained, and rectal temperature recorded to determine clinical responses and ST shedding. Bodyweight and feed intake were recorded on d 0, 7, and 21 post-inoculation to estimate ADG, ADFI, and G:F. Rectal temperature increased ($P < 0.05$) 24 h post-inoculation and remained elevated at d 6. Serum albumin concentration decreased ($P < 0.05$), whereas haptoglobin concentration increased ($P < 0.05$) post- ST inoculation. There was no fibre or Thr effect ($P > 0.05$) on ST counts in the ileum and cecum, but a fibre \times Thr interaction ($P < 0.05$) was observed in the colon. Supplemental Thr increased ($P < 0.05$) growth performance in LF- and HF-fed challenged pigs. However, the growth performance response of supplemented HF challenged pigs was less than supplemented LF challenged pigs. These results suggest that Thr supplemented to meet requirements for high DF and the systemic immune challenge was not sufficient to maintain growth performance of pigs fed HF diets and challenged with an enteric pathogen.

6.2 Introduction

Optimal growth performance can be affected by a wide-range of factors in the production system of growing-finishing pigs. For instance, factors such as subclinical disease challenges, inappropriate levels of essential nutrients such as AA, and the negative effects of high fibre ingredients, which are poorly digestible (Stein et al., 1999) can adversely affect pig performance. Dietary fibre (**DF**) has been reported to independently increase the threonine (**Thr**) requirement for maximum growth and PD in growing pigs (Mathai et al., 2016; Wellington et al., 2018). Similarly, Blank et al. (2012) and Libao-Mercado et al. (2006; 2007) have reported fibre-associated Thr losses in growing pigs and concluded that those effects were due to high endogenous loss of mucins. Commercial production systems are likely to predispose pigs to subclinical disease, which may affect the immune system function. Immune system stimulation has been reported to partition nutrients away from growth to support the immune response (Reeds et al., 1994; Reeds and Jahoor, 2001) and has been shown to increase maintenance energy (Huntley et al., 2018) and AA requirements (Litvak et al., 2013a; Rakhshandeh et al., 2014; Wellington et al., 2018) in pigs. In contrast, Rudar et al. (2016) reported no effect of feeding supplemental leucine on nitrogen retention in starter pigs challenged with *E. coli* lipopolysaccharide. In a previous study, we demonstrated that high DF and ISS independently increased the Thr requirement to maximize growth performance and PD (Wellington et al., 2018). Interestingly, when both high DF and a systemic immune challenge were present, no further increase in the Thr requirement for PD was observed. Thus, ISS and DF effects on Thr requirement were not additive.

Since systemic LPS challenge may have limited direct effects on the gastrointestinal tract, the objective of the current study was, therefore, to investigate whether supplementing Thr to meet high DF and systemic immune challenge requirements would maintain the performance of pigs exposed to an enteric immune challenge when fed high DF.

6.3 Materials and Methods

The experimental protocol was reviewed and approved by the University of Saskatchewan's Animal Research Ethics Board (Animal Use Protocol #20170123) and followed the Canadian Council on Animal Care guidelines (CCAC, 2009)

6.3.1 Animals, Housing and Diets

The experiment duration was 28-d and consisted of a 7-d adaptation period (no challenge) and 21 d post-inoculation period. A total of 128 pigs (Camborough Plus × C3378; PIC, Canada, Ltd.) of 22.6 ± 1.6 kg initial BW were obtained from the Prairie Swine Centre, Inc. (Saskatoon, SK) and transported to the Animal Care Unit of the Western College of Veterinary Medicine (Saskatoon, SK). The pigs were placed on trial in 2 blocks using 2 experimental rooms for each block. In each experimental room (22 ± 1 °C temperature), pigs were housed in groups of 4 pigs/pen on solid floors lined with rubber mats. Pens were randomly assigned to 1 of 4 dietary treatments in a 2×2 factorial arrangement in a randomized complete block design (n=8 pens/treatment). Treatments consisted of a low (**LF**; 13% TDF) or high (**HF**; 20% TDF) fibre diet with either a standard (STD; 0.65% SID) or supplemental (SUP; 0.78% SID) Thr level. The SUP Thr level was based on previous studies examining the effects of systemic ISS and DF on Thr requirements in growing pigs (Wellington et al., 2018; 2019a).

Diets were wheat- and soybean-based and were formulated using the analyzed contents of AA and published SID coefficients of AA for ingredients to meet or exceed nutrient requirements for 25-50 kg pigs according to NRC (2012) and Evonik (AMINODat® 5.0). The HF diets were formulated by partly replacing corn in the LF diet with 5% wheat bran and 10% sugar beet pulp (Table 6.1). Pigs were fed *ad libitum* and had unrestricted access to water. Samples of experimental diets were collected during the trial and ground in a centrifugal mill (ZM 100, RETSCH GmbH & Co., Rheinische Straße, Germany) to pass through a 1 mm sieve. The diets were analyzed for AA contents via ion-exchange chromatography with post-column derivatization with ninhydrin (Evonik Nutrition & Care GmbH, Hanau, Germany; Llamas and Fontaine, 1994). The dry matter (DM) content of the diets was determined according to AOAC (2007) method 930.15. Nitrogen (N) content of the experimental diets (Table 6.2) was determined using an automatic analyser (LECO FP 528; MI, USA; Method 990.03; AOAC 2007) and crude protein

(CP) content of the diets calculated as $N \times 6.25$. Total dietary fibre (TDF), soluble dietary fibre (SDF), and insoluble dietary fibre (IDF) of the experimental diets were analyzed according to the AOAC (2007) method 991.43 using an ANKOM^{TDF} analyser (ANKOM Technology, Macedon NY, USA). All analyses were conducted in duplicate. Individual pig BW and feed disappearance were measured on d 0, 7, and 21 post-inoculation for calculation of average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency (G:F).

Table 6.1 Composition of experimental diets (as-fed basis) for low and high fibre with standard or supplemental threonine

<i>Ingredients, %</i>	Low fibre		High fibre	
	STD Thr¹	SUP Thr²	STD Thr	SUP Thr
Corn	21.84	21.72	3.96	3.83
Wheat	47.0	47.0	47.0	47.0
Barley	10.0	10.0	10.0	10.0
Wheat bran	-	-	5.0	5.0
Sugar beet pulp	-	-	10.0	10.0
Canola oil	0.90	0.90	3.80	3.80
Soybean meal	17.0	17.0	17.0	17.0
L-Lys-HCl	0.53	0.52	0.50	0.50
DL-Met	0.20	0.20	0.20	0.20
L-Thr	0.15	0.28	0.16	0.29
L-Trp	0.03	0.03	0.03	0.03
L-Val	0.05	0.05	0.05	0.05
Limestone	1.25	1.25	1.25	1.25
Monocalcium phosphate	0.75	0.75	0.75	0.75
Salt	0.20	0.20	0.20	0.20
Vitamin-mineral premix ³	0.10	0.10	0.10	0.10
<i>Calculated nutrient content</i>⁴				
DM, %	87.4	87.4	84.7	84.7
CP, %	17.3	17.4	17.5	17.6
ME, kcal/kg	3285	3287	3318	3317
NE, kcal/kg	2488	2489	2486	2484
<i>Amino Acids, % SID⁵</i>				
Arg	0.92	0.92	0.92	0.92
His	0.40	0.40	0.39	0.39
Ile	0.60	0.60	0.59	0.59
Leu	1.15	1.15	1.07	1.07
Lys	1.08	1.08	1.08	1.08
Met	0.44	0.44	0.42	0.42
Cys	0.29	0.29	0.30	0.30
Phe	0.74	0.74	0.72	0.72
Tyr	0.45	0.45	0.46	0.46
Thr	0.65	0.78	0.65	0.78
Trp	0.22	0.22	0.23	0.23
Val	0.73	0.73	0.73	0.73

¹STD Thr, Standard Thr (0.65% standardized ileal digestible).

²SUP Thr, Supplemental Thr (0.78% standardized ileal digestible).

³Supplied per kg of complete diet: vitamin A, 8000 IU; vitamin D, 1500 IU; vitamin E, 30 IU; menadione, 2.5 mg; vitamin B12, 0.025 mg; thiamine, 1.00 mg; biotin, 0.10 mg; niacin, 20 mg; riboflavin, 4 mg; pantothenate, 12 mg; folic acid, 0.50 mg; pyridoxine, 2.0 mg; Fe, 100 mg; Zn, 100 mg; Mg, 40 mg; Cu, 15 mg; Se, 0.30 mg and I, 1 mg.

⁴Nutrient content of diets based on estimated nutrient contents of ingredients according to NRC (2012). ⁵SID, Standardized ileal digestible

Table 6.2 Analyzed nutrient content of experimental diets (as-fed basis), with low or high fibre with standard or supplemental threonine.

<i>Item, %</i>	Low fibre		High fibre	
	STD Thr¹	SUP Thr²	STD Thr	SUP Thr
DM	89.20	90.10	90.60	90.10
CP	18.57	18.54	18.13	18.60
SDF ³	1.80	1.90	3.60	3.70
IDF ⁴	11.20	11.80	17.30	16.10
TDF ⁵	13.00	13.70	20.90	19.80
<i>Total amino acids⁶</i>				
Arg	1.07 (0.99)	1.04 (0.99)	1.07 (0.99)	1.08 (0.99)
His	0.43 (0.40)	0.43 (0.40)	0.44 (0.41)	0.45 (0.41)
Ile	0.70 (0.64)	0.68 (0.64)	0.70 (0.64)	0.70 (0.64)
Leu	1.31 (1.23)	1.29 (1.23)	1.24 (1.14)	1.25 (1.14)
Lys	1.22 (1.15)	1.22 (1.15)	1.26 (1.16)	1.23 (1.16)
Met	0.46 (0.45)	0.47 (0.45)	0.47 (0.44)	0.45 (0.44)
Cys	0.34 (0.29)	0.34 (0.29)	0.33 (0.31)	0.33 (0.31)
Phe	0.86 (0.77)	0.84 (0.77)	0.84 (0.76)	0.85 (0.76)
Thr	0.78 (0.72)	0.87 (0.85)	0.78 (0.72)	0.87 (0.85)
Trp	0.26 (0.23)	0.25 (0.23)	0.26 (0.24)	0.26 (0.24)
Val	0.85 (0.77)	0.84 (0.77)	0.86 (0.78)	0.86 (0.78)

¹STD Thr, Standard Thr (0.65% standardized ileal digestible).

²SUP Thr, Supplemental Thr (0.78% standardized ileal digestible).

³SDF, Soluble dietary fibre.

⁴IDF, Insoluble dietary fibre.

⁵TDF, Total dietary fibre.

⁶Analyzed values of total amino acids with calculated values in brackets.

6.3.2 Inoculation and Rectal Swab Protocol

On d 0 of the challenge period (d 8 of the trial), pigs were orally inoculated twice within 4 h, each time with 1 mL solution containing 2.3×10^9 CFU/mL of *Salmonella typhimurium* var Copenhagen selected for antibiotic resistance to Novobiocin and Nalidixic acid (Pieper et al., 2009). Rectal temperatures were obtained from all pigs on d 2 pre-inoculation and every 24 h post-inoculation for 6 d using a digital thermometer (Life Brand, ON, Canada). On d 2 pre-inoculation and d 1, 2, 4, 6, 14, and 20 post-inoculation, rectal swabs were obtained from individual pigs, diluted 1:10 in buffered peptone water (**BPW**) and cultured on brilliant green agar (BG agar) plates containing 30 µg/mL Nalidixic acid and 50 µg/mL Novobiocin (Nal+/Nov+). Further, 1 mL of the dilution was enriched in 4 mL of selenite-cysteine broth containing 30 µg/mL Nalidixic acid and 50 µg/mL Novobiocin and incubated overnight at 37 °C and later cultured on BG agar plates (Nal+/Nov+). Colony counts were recorded on all plates after incubation for 24 h at 37° C. A scoring system was used to assign fecal shedding scores for each swab (Burkey et al., 2004). Plates prepared from swabs with colony counts > 30 were given a shedding score of 3. Plates positive for antibiotic-resistant ST but with colony counts < 30 were given shedding score 2. A shedding score of 1 was assigned to swabs that were negative for ST following direct plating but positive after enrichment. Finally, swabs negative for antibiotic-resistant ST following direct plating and on enrichment were given a score of zero.

6.3.3 Blood Sampling and Analyses

Blood samples were obtained from 2 pigs/pen one day before the ST inoculation and on d 4 and 7 post-inoculation. Blood samples were collected via jugular vein puncture into 10 mL vacutainer tubes containing either EDTA or no additive (BD, Vacutainers Mississauga, ON, Canada). Whole blood samples collected in EDTA tubes were immediately submitted and analyzed for complete blood cell count at Prairie Diagnostic Services (Saskatoon, SK) using an ADVIA 2120i haematology analyser (Siemens Health Care Diagnostics, Deerfield, IL). Blood samples collected into additive-free tubes were allowed to clot and then centrifuged at $2500 \times g$ at 4 °C for 15 min.

Serum samples were obtained and stored at -20 °C for subsequent analysis for albumin, haptoglobin, and IgG. Briefly, serum albumin was analyzed by bromocresol green method using a Cobas C 311 (Roche Diagnostics, Laval, QC, Canada) according to Doumas et al. (1971). Serum IgG levels were determined by radial immunodiffusion assay as previously described by Chelack et al. (1993) with antiserum incorporated into the gel: goat anti-swine IgG (H+L; Jackson Immuno Research Laboratories, Inc., West Grove, PA). A porcine reference serum was used for a standard curve supplied by Bethyl Laboratories, Inc., (Montgomery, TX). A secondary reference serum was used as a control assay (Immuno-Reagents, Raleigh, NC). Serum haptoglobin was analyzed in the Animal Health Laboratory (University of Guelph, Guelph, ON) according to a method described by Makimura and Suzuki (1982) on a Roche Cobas 6000 c501 analyser.

6.3.4 Tissue and Digesta Collection and Analyses

On d 7 post-inoculation, one pig/pen representing the average pen BW was humanely euthanized by penetrating captive bolt followed by exsanguination. Subsequently, mesenteric lymph nodes (**MLN**) and spleen were sampled under aseptic conditions into sterile tubes containing 20 mL BPW and 200 µL of the dilution was plated on Nal+/Nov+ BG agar plates. Further, 1 mL was diluted in 4 mL selenite-cysteine broth (Nal+/Nov+) for enrichment overnight. The enriched samples were incubated overnight at 37 °C with shaking, after which 200 µL was plated and incubated. A shedding score was assigned as previously described. Intestinal digesta samples (~1 g) were obtained from the ileum, cecum, and colon and each diluted in 4 mL BPW and kept at 4 °C. The digesta samples were serially diluted to 10^{-7} and 200 µL of each dilution was plated on BG agar (Nal+/Nov+) and cultured at 37 °C for 24 h after which colonies were counted on each plate and recorded. Colony counts of 30-300 were used in the calculations of colony-forming units per gram digesta (CFU/g).

6.3.5 Statistical Analyses

Data were tested for normality and outliers were verified using the PROC UNIVARIATE model in SAS (version 9.4, SAS Institute Inc., Cary, NC) and the Shapiro-Wilk test. Outliers were determined as a value ± 2 standard deviations away from the treatment mean using the studentized residual analysis. All data were analyzed as a 2×2 factorial in a randomized complete block design (PROC MIXED) with main effects of a) fibre level, b) threonine level and their interactions. A block was included in the model as a random effect. For blood data, the period was also included as a fixed effect in the statistical model and run as a repeated measure analysis. The Tukey-Kramer mean separation test was used to determine significant differences, LSMEANS considered significant at $P < 0.05$ and a trend toward significance considered at $P > 0.05$ and $P \leq 0.10$.

6.4 Results

6.4.1 Response to *Salmonella typhimurium* Inoculation

The response of pigs to ST inoculation was determined by measuring rectal temperature over a period of 6 d post-inoculation after an initial measurement prior to the oral inoculation. There was an increase ($P < 0.01$) in rectal temperature on d 1 to 6 compared to the pre-inoculation levels (Fig. 6.1). There was no significant ($P > 0.05$) period effect on white blood cell count (WBC); however, HF reduced WBC count ($P < 0.05$) whereas SUP Thr tended ($P = 0.08$) to reduce the WBC count (Table 6.3). The period had a significant ($P < 0.01$) effect on serum albumin. Specifically, there was a reduction ($P < 0.01$) in serum albumin concentration on d 4 post-inoculation compared to pre-inoculation, which returned to pre-inoculation levels ($P > 0.05$) on d 7 post-inoculation (Table 6.3). Fibre had no effect ($P > 0.05$) on serum albumin concentration but SUP Thr tended ($P = 0.06$) to reduce serum albumin concentration. Period (pre-inoculation, d 4 post-inoculation and d 7 post-inoculation) had a significant effect ($P < 0.01$) on serum haptoglobin concentration. Specifically, haptoglobin levels increased on d 4 post-inoculation and remained elevated on d 7 compared to the pre-inoculation levels. There were no significant effects ($P > 0.05$) of fibre or Thr levels on serum haptoglobin and serum IgG concentrations (Table 6.3).

6.4.2 *Salmonella typhimurium* Shedding

Dietary treatment had no effect ($P > 0.05$) on ST count in the caecum (Table 6.4). In the ileum, there were tendencies for fibre ($P = 0.06$) and SUP Thr ($P = 0.06$) to increase ST count. In the colon, a significant fibre \times Thr interaction ($P < 0.01$) was observed where SUP Thr decreased ST counts in pigs fed HF diets but not in LF-fed pigs (Table 6.4). Although ST was present in all pigs (feces, digesta, and tissue samples), there were no significant effects ($P > 0.05$) of fibre, Thr or fibre \times Thr interaction on ST shedding in feces (Fig 6.2). Analysis for the effect of sampling day on fecal shedding, show a decline in the mean scores from d 1 to 20 post-ST inoculation. There were no significant effects ($P > 0.05$) of fibre, Thr, or fibre \times Thr interaction on ST in the MLN and spleen of pigs (Fig 6.3).

6.4.3 *Growth Performance Post Salmonella typhimurium* Inoculation

In the first week (d 0-7) of the ST challenge period, there was a significant effect of fibre level ($P < 0.01$; Table 6.5) on ADG. Feeding HF diets reduced ADG compared to LF (0.877 vs. 1.023 kg/d). There was also a significant effect of Thr ($P < 0.01$) level on ADG, where SUP Thr increased ADG compared to STD Thr level (0.99 vs. 0.90 kg/d). Similarly, there were both significant fibre ($P < 0.01$) and Thr ($P < 0.01$) effects on ADFI, such that ADFI was higher in the LF fed pigs compared to the HF-fed pigs (1.71 vs. 1.60 kg/d). Supplemental Thr reduced the ADFI (1.59 kg/d) compared to the STD Thr fed pigs (1.72 kg/d). For both ADG and ADFI no significant interaction ($P > 0.05$) between fibre and Thr was observed.

In the present study, we observed an effect of Thr level on pig performance during week 1 (d 0-7) and weeks 2 and 3 (d 8-21) post-inoculation independent of DF level. During d 0-7 post-inoculation, pigs fed SUP Thr had lower ADFI (1.593 kg/d) but higher ADG (0.996 kg/d) and G:F (0.63 kg/kg) compared to pigs fed the STD Thr diets although they recorded a higher ADFI (1.720 kg/d). These levels of feed intake resulted in daily Thr intake of 13.5 and 12.4 g/d in SUP Thr and STD Thr-fed pigs, respectively. Based on the observed significant effects of both DF and Thr on ADG and ADFI, G:F also showed a significant effect for fibre ($P = 0.02$) and Thr ($P < 0.01$) with no significant interaction. A lower G:F was observed in HF-fed pigs compared to the LF fed pigs

(0.55 vs. 0.60 kg/kg) and SUP Thr improved G:F compared to STD Thr level (0.63 vs. 0.53 kg/kg). From d 8-21 post-inoculation, no significant effect ($P > 0.05$) of fibre, Thr or interaction on ADFI was observed. There were significant effects of fibre ($P < 0.01$) and Thr ($P < 0.01$) on ADG, with no significant interaction. High fibre reduced ADG (0.87 vs. 1.02 kg/d) compared to the LF fed pigs while SUP Thr improved ADG compared with the STD Thr fed pigs, regardless of fibre level. Feed efficiency during this period showed significant fibre ($P < 0.01$) and Thr ($P < 0.01$) effects. The HF-fed pigs had a reduced G:F (0.51 vs. 0.56) compared to the LF fed pigs. Also, feeding SUP Thr improved the G:F as compared with the STD Thr fed pigs. Overall (d 0-21 post-inoculation), pigs fed SUP Thr had higher ADG (1.01 vs. 0.88 kg/d), lower ADFI (1.74 vs. 1.84 kg/d), and higher G:F (0.58 vs 0.49) than STD Thr group ($P < 0.05$). Similarly, pigs fed LF diets had higher ADG (1.02 vs. 0.87 kg/d), ADFI (1.83 vs. 1.74 kg/d), and G:F (0.56 vs 0.51 kg/kg) than HF-fed pigs ($P < 0.05$).

Table 6.3 Plasma parameters of immune status in pigs inoculated with *Salmonella typhimurium*

	Low fibre		High fibre			P-value ⁴		
Item	STD Thr ¹	SUP Thr ²	STD Thr	SUP Thr	SEM ³	Fibre	Thr	Period
<i>White blood cell counts, ×10⁹, g/L</i>								
Pre-Inoculation	20.32	18.98	18.86	16.36				
Post-Inoculation (d 4)	19.93	20.41	19.46	17.51	1.03	<0.05	0.08	0.43
Post-Inoculation (d 7)	19.41	20.37	20.17	18.23				
<i>Albumin, g/L</i>								
Pre-Inoculation	35.92	36.50	37.63	36.00				
Post-Inoculation (d 4)	30.08	29.47	31.00	27.60	1.59	0.87	0.07	<0.01
Post-Inoculation (d 7)	36.31	34.89	37.63	34.24				
<i>Haptoglobin, g/L</i>								
Pre-Inoculation	0.94	1.26	1.22	0.96				
Post-Inoculation (d 4)	1.71	1.73	1.56	1.56	0.27	0.74	0.82	<0.01
Post-Inoculation (d 7)	1.66	1.89	1.87	1.75				
<i>Immunoglobulin G, mg/L</i>								
Pre-Inoculation	10.27	11.09	10.42	10.94				
Post-Inoculation (d 4)	11.24	11.79	11.5	11.52	1.27	0.73	0.41	0.38
Post-Inoculation (d 7)	10.44	12.39	12.38	11.85				

¹STD Thr, Standard Thr level (0.65% standardized ileal digestible).²SUP Thr, Supplemental Thr level (0.78% standardized ileal digestible; 20% above STD Thr).³SEM, Standard error of the mean.⁴No significant two- or three-way interactions were observed.

Table 6.4 *Salmonella typhimurium* quantification in intestinal contents (Log10 CFU/g; d 7 post-inoculation) of inoculated pigs

<i>Item</i>	Low fibre		High fibre		SEM³	<i>P-value</i>		
	STD Thr¹	SUP Thr²	STD Thr	SUP Thr		<i>Fibre</i>	<i>Thr</i>	<i>Fibre × Thr</i>
Ileum	4.55	4.94	4.96	6.43	0.49	0.06	0.06	0.26
Cecum	4.39	4.96	4.94	4.22	0.48	0.87	0.84	0.19
Colon	5.21	6.84	7.03	4.81	0.73	0.66	0.86	<0.01

¹STD Thr, Standard Thr level (0.65% standardized ileal digestible).

²SUP Thr, Supplemental Thr level (0.78% standardized ileal digestible; 20% above STD Thr).

³SEM, Standard error of the mean.

Table 6.5 Growth performance of pigs inoculated with *Salmonella typhimurium*

<i>Parameters</i>	Low fibre		High fibre		SEM³	<i>P-value</i>⁴	
	STD Thr¹	SUP Thr²	STD Thr	SUP Thr		<i>Fibre</i>	<i>Thr</i>
Initial BW, kg	22.76	22.32	22.69	22.45	0.167	0.86	0.15
Final BW, kg	49.51	51.45	46.34	48.57	1.055	<0.01	<0.01
<i>Pre-Inoculation (d -7-0)</i>							
ADG, kg	0.826	0.804	0.818	0.772	0.043	0.42	0.18
ADFI, kg	1.339	1.359	1.218	1.185	0.089	<0.01	0.23
G:F, kg/kg	0.620	0.595	0.636	0.655	0.020	<0.01	0.81
<i>Post-Inoculation (d 0-7)</i>							
ADG, kg	0.965	1.082	0.843	0.911	0.025	<0.01	<0.01
ADFI, kg	1.788	1.635	1.653	1.551	0.047	<0.01	<0.01
G:F, kg/kg	0.543	0.665	0.511	0.588	0.022	<0.05	<0.01
<i>Post-Inoculation (d 8-21)</i>							
ADG, kg	0.940	1.093	0.783	0.938	0.031	<0.01	<0.01
ADFI, kg	1.983	1.944	1.934	1.831	0.075	0.29	0.36
G:F, kg/kg	0.483	0.566	0.409	0.514	0.022	<0.01	<0.01
<i>Overall (d 0-21)</i>							
ADG, kg	0.953	1.088	0.813	0.924	0.020	<0.01	<0.01
ADFI, kg	1.885	1.789	1.793	1.691	0.042	<0.05	<0.05
G:F, kg/kg	0.514	0.615	0.459	0.553	0.017	<0.01	<0.01

¹STD Thr, Standard Thr level (0.65% standardized ileal digestible).²SUP Thr, Supplemental Thr level (0.78% standardized ileal digestible).³SEM, Standard error of the mean.⁴No significant Fibre × Thr interactions were observed.

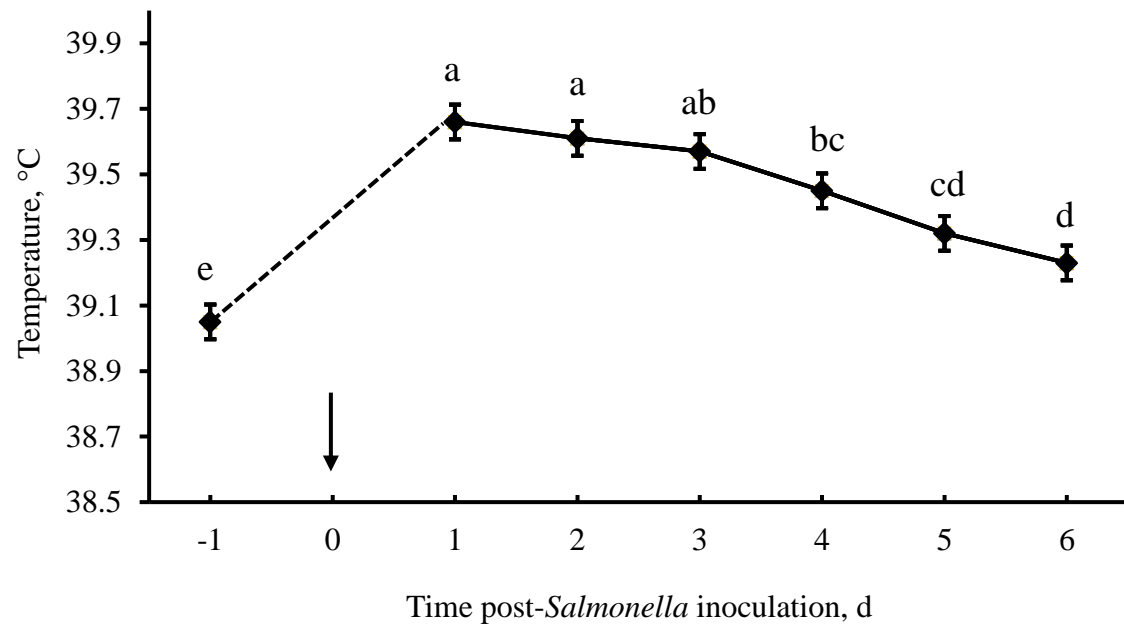


Figure 6.1 Rectal temperature (°C) of pigs prior to *Salmonella typhimurium* inoculation and monitored for 6 d post-inoculation. The arrow indicates time point of *Salmonella typhimurium* inoculation.

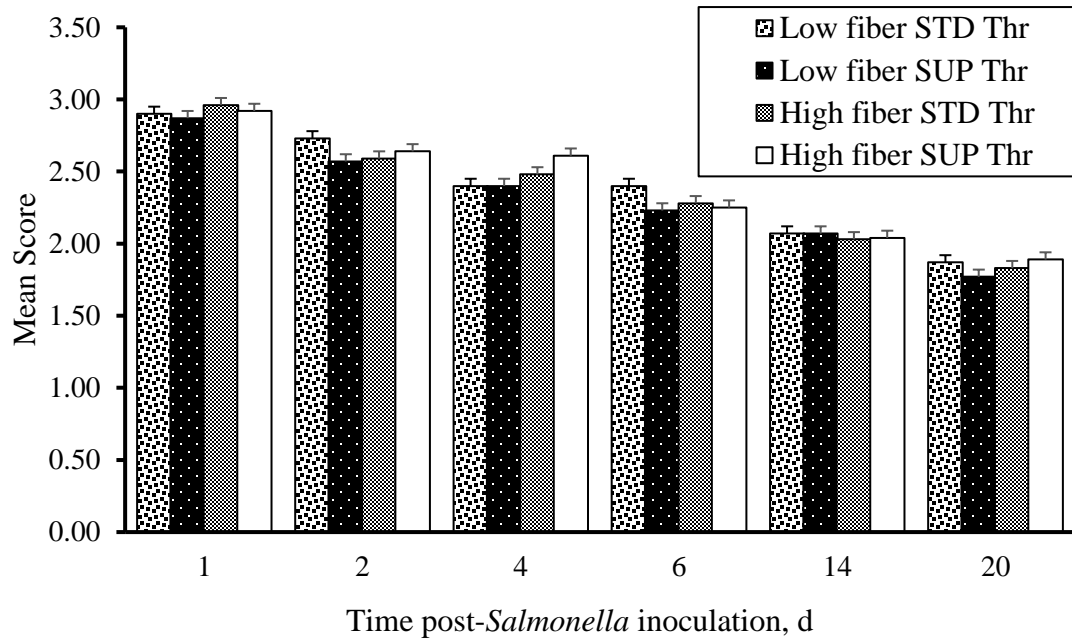


Figure 6.2 Post-inoculation fecal shedding of *Salmonella typhimurium* on d 1, 2, 4, 6, 14 and 20. Data presented as mean scores. A shedding score of 3 was assigned to plates positive for the inoculated *Salmonella typhimurium* with counts > 30 and plates positive but with counts < 30 were given shedding score of 2. A shedding score of 1 was assigned to plates that were only positive after enrichment and plates negative after enrichment were scored zero. No significant ($P > 0.05$) fibre, threonine or interactive effects on *Salmonella typhimurium* shedding on d 1, 2, 4, 6, 14 and 20 was observed. High fibre diets [HF; 20% total dietary fibre] and low fibre diets [LF; 13% total dietary fibre]. Standard threonine (STD Thr; 0.65% SID) and supplemental threonine (SUP Thr; 0.78% SID).

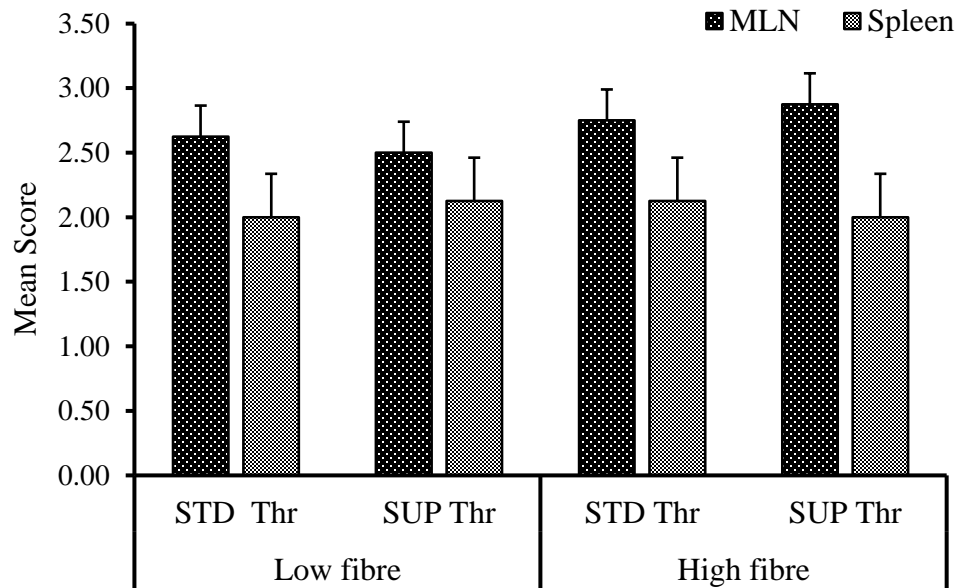


Figure 6.3 *Salmonella typhimurium* translocation to the mesenteric lymph nodes (MLN) and spleen. Data presented as mean scores. A shedding score of 3 was assigned to plates positive for the inoculated *Salmonella typhimurium* with counts > 30 and plates positive but with counts < 30 were given shedding score of 2. A shedding score of 1 was assigned to plates that were only positive after enrichment and plates negative after enrichment were scored zero. We observed no significant ($P > 0.05$) effect of fibre, threonine or interactions on shedding in either MLN or spleen. High fibre diets [HF; 20% total dietary fibre] and low fibre diets [LF; 13% total dietary fibre]. Standard threonine (STD Thr; 0.65% SID) and supplemental threonine (SUP Thr; 0.78% SID).

6.5 Discussion

The objective of the present study was to determine if growth performance of pigs fed high DF and challenged with an enteric pathogen would be maintained when Thr supply was adjusted to meet previously estimated requirements for both high DF and systemic immune challenge (Wellington et al., 2018; 2019a). We reported previously that high DF and systemic immune challenge with *E. coli* LPS independently increased the Thr requirement for maximum PD (Wellington et al., 2018). In the present study, we fed the same level of DF with a standard level (0.65% SID; NRC 2012) or supplemental level (0.78% SID) of Thr. The SUP Thr (0.78% SID) was previously determined to be enough to maximize growth performance (Wellington et al., 2019b) and PD (Wellington et al., 2018) when feeding the same high DF as used in the present study. However, when a systemic immune challenge was applied and high DF was fed, we estimated 0.76% SID Thr required to maximize PD. Therefore, the supplemental Thr level (0.78% SID) used in the present study was expected to be adequate to mount an immune response and support growth (Wellington et al., 2018).

6.5.1 Response to *Salmonella typhimurium* Inoculation

The enteric pathogen model (*Salmonella typhimurium*) used in the current study has been reported to successfully and uniformly stimulate the immune system of pigs (Pieper et al., 2012; Barba-Vidal et al., 2017; Burdick-Sanchez et al., 2017). In the present study, the increase in haptoglobin and decrease in serum albumin post-inoculation agrees with response observed in previous studies in pigs under immune stimulation (Lipperheide et al., 1998; Petersen et al., 2002; de Ridder et al., 2012; Litvak et al., 2013a). Turner et al. (2002) and Gebru et al. (2010), who measured rectal temperature in pigs for 7 d post-*Salmonella* inoculation and reported the highest rectal temperatures on d 2 and 3, which remained elevated until d 7, comparable to the response seen in the current study. Previous studies in which LPS was used to initiate an immune stimulation observed alterations in WBC counts (Litvak et al., 2013a; Wellington et al., 2018). This was not observed and may be due to differences in the infection model used (e.g., enteric pathogen vs. systemic LPS) or time of blood sampling. The lack of response of serum IgG to ST inoculation agrees with Turner et al. (2002), who reported no significant effect of the ST challenge model on

serum IgG levels in weanling pigs. However, others have reported that increasing dietary Thr levels increased serum IgG concentration in growing pigs (Li et al., 1999). Altogether, the data suggest an effective and uniform ST challenge across all the dietary treatments. Rectal swabs collected prior to ST inoculation confirmed that pigs used in the current study were negative for the inoculated strain of ST. *Salmonella typhimurium* shedding in feces was detected in all pigs until d 20 following ST inoculation, consistent with prolonged inflammatory indicators.

Furthermore, ST was detected in digesta and tissue samples on day 7 post-inoculation indicating intestinal colonization and translocation of the ST. Dietary treatment did not affect fecal shedding which agrees with Thompson et al. (2012) who fed diets containing different fibre sources (sugar beet pulp, distiller dried grains, and soluble) to pigs inoculated with ST and observed no significant changes in fecal ST shedding. In contrast, others have reported changes in *Salmonella* colonization which has been associated with variations in barley fibre (Pieper et al., 2012). No significant treatment effects were observed on ST count in digesta (ileum and cecum), MLN, and spleen tissues, consistent with the observations made on fecal shedding scores. In the colon digesta however, interaction of fibre and Thr on shedding was observed, suggesting that supplemental Thr may have supported immune responses contributing to reduced ST colonization when HF diets were fed, however, given this observation limited to the colon.

6.5.2 Growth Performance of *Salmonella typhimurium* Inoculated Pigs

We observed no significant interactions between DF and Thr level on growth performance and therefore only main effects are discussed here. In the present study, the observed effect of HF diets (54% increase in TDF content) on growth performance was consistent during the first week (d 0-7) post-inoculation, weeks 2 and 3 (d 8-21) post-inoculation, and overall (d 0-21). In the first week post-inoculation, the reduced ADG observed in HF-fed pigs is likely due to lower ADFI as well as reduced feed efficiency. In the post-inoculation period d 8-21, although all pigs had the same ADFI, ADG and G:F were reduced in HF-fed pigs. The fibrous ingredients (sugar beet pulp and wheat bran) that were used to produce the HF diet in the present study have been reported by others (Anguita et al., 2007) to reduce feed intake in pigs thereby reducing growth rate and efficiency in the absence of a pathogenic challenge. However, in the current study dietary nutrient

content (net energy and amino acids) was formulated to be consistent across dietary treatments and, therefore, should not have resulted in reduced performance.

Under poor sanitary conditions, the addition of fibre (16.9% total DF) in pig diets has been reported to negatively affect growth performance by decreasing voluntary feed intake (Montagne et al., 2012). Similarly, when pigs were inoculated with *Salmonella enterica* serovar Typhimurium, voluntary feed intake decreased compared to pre-inoculation intake levels, suggesting that the presence of a disease challenge also affects voluntary feed intake (Gebru et al., 2010). This is likely why HF diets, despite formulation to similar nutrient levels as LF diets, reduced feed intake and resulted in decreased growth performance in the current study. It has been suggested that during enteric pathogen challenge, DF may act as a substrate for increased bacterial proliferation and hence increased disease prevalence and poor performance of pigs (Montagne et al., 2012). Nevertheless, there was no evidence of increased ST colonization with HF diets in the current study. Factors contributing to lower intake and performance during enteric disease challenge are unclear but appear to be unrelated to differences in ST colonization or supply of Thr. Under immune-stimulating conditions, such as disease challenge or poor sanitary conditions, nutrient requirements increase to support an effective immune response (Le Floch et al., 2009; Van der Meer et al., 2016). Threonine has been reported to support optimal growth, immune function and intestinal barrier in pigs when supplied at the required levels (Wang et al., 2010; Mao et al., 2014), and play important roles in mucin and immunoglobulin production (Trevisi et al., 2015). The addition of supplemental Thr above the recommended level for growth has been reported to improve weight gain and feed efficiency in growing pigs (Li et al., 1999). Other reports have suggested that higher Thr levels are required to optimize growth performance under unsanitary conditions (Jayaraman et al., 2015) and during systemic ISS with LPS (Wellington et al., 2018; McGilvray et al., 2019a).

Supplementation of dietary Thr improved ADG regardless of DF and resulted in similar effects as high DF on ADFI and G:F during all post-inoculation periods in the current study. It is unclear why SUP Thr decreased feed intake post-inoculation. Although it is possible the SUP Thr increased susceptibility to ST challenge there is little evidence in support of this on recorded clinical responses, salmonella colonization and translocation in tissues. Moreover, Trevisi et al. (2015) reported that feed intake was increased in pigs (8-10 kg BW) that were challenged with *E.*

coli and fed relatively high Thr diets (0.90%) compared to pigs fed lower Thr diets (0.85%) during d 0-7 post-inoculation. Regardless of the effects on feed intake, in the current study, supplementing Thr above NRC (2012) requirements for growth resulted in improved ADG and G:F. The improved growth performance observed in the present study agrees with Trevisi et al. (2015) who reported an improved ADG and G:F in pigs fed high Thr diets and challenged with *E. coli*. Similarly, Ren et al. (2014) reported improved growth performance (ADG and G:F) and intestinal immune status when higher Thr was fed to weaned pigs challenged with *E. coli*. Therefore, under conditions of disease challenge where pigs are unable to maintain voluntary feed intake at required levels to meet energy and nutrient requirement for an effective immune response and growth, increased dietary Thr could play an important role in alleviating the effects of the disease challenge by maintaining growth performance and immune status.

It is noteworthy that while feeding high DF in the present study reduced pig growth performance during an enteric disease challenge and supplemental Thr improved pig performance regardless of the DF content, there was no interaction between Thr supplementation and DF. So, while SUP Thr improved growth performance in challenged pigs fed high DF diets, this growth response was less than the response in LF-fed pigs. This suggests that the level of Thr supplementation used in this study was insufficient to meet requirements in pigs fed high DF and challenged with an enteric pathogen or another factor is limiting for growth under the conditions of the current study. This contrasts with our previous work (Wellington et al., 2018) in which we demonstrated that both high DF and ISS independently increased Thr requirement for PD but did not result in a further increase when both were present (i.e., no additive effect). It has been shown that challenge models (e.g., enteric pathogen, LPS challenge, sanitary conditions) can result in different effects on immune response (Goodband et al., 2014) and caution should, therefore, be used when comparing and interpreting results of studies in which different models have been used.

6.6 Conclusion

Overall, supplementing dietary Thr above the estimated requirement (NRC, 2012) improved growth performance in LF- and HF-fed challenged pigs. However, the performance of supplemented HF challenged pigs was less than supplemented LF challenged pigs. These results suggest that Thr supplementation to meet requirements for high DF and a systemic immune challenge was not sufficient to maintain growth performance of pigs fed HF diets and challenged with an enteric pathogen.

6.7 Acknowledgements

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CHAPTER 7
**EFFECT OF DIETARY FIBRE AND THREONINE CONTENT ON INTESTINAL
BARRIER FUNCTION IN PIGS CHALLENGED WITH EITHER SYSTEMIC *E. COLI*
LIPOPOLYSACCHARIDE OR ENTERIC *SALMONELLA TYPHIMURIUM*⁷**

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Author Contributions

DAC, AVK, MOW and JKH conceived and designed the experiment. MOW, KH, JECK carried out the experiment. MOW analyzed all data and drafted the manuscript. All authors read, edited and approved the final manuscript.

7.1 Abstract

The independent and interactive effects of dietary fibre (**DF**) and Thr on intestinal barrier permeability and function was investigated using different markers of barrier function under conditions of either a systemic *E. coli* lipopolysaccharide (**LPS**) challenge (Experiment 1) or an enteric *Salmonella typhimurium* (**ST**) challenge (Experiment 2) to evaluate the impact of different immune challenge models. In experiment 1, 90 barrows (20.5 ± 0.75 kg initial body weight) were randomly assigned to a low fibre (**LF**; 12.5% total DF) or high fibre (**HF**; 18.5% total DF) diet with graded levels of Thr (0.49, 0.57, 0.65, 0.73 and 0.81% standardized ileal digestible [**SID**]). Immune system stimulation (**ISS**) was induced by repeated injections of *E. coli* LPS. Intestinal permeability was assessed in the pre-ISS and ISS periods via oral gavage with a lactulose solution (0.67 g/mL lactulose) and mannitol solution (0.43 g/mL mannitol) to provide 0.5 g/kg BW and 0.3 g/kg BW lactulose and mannitol, respectively. Total urine output was collected over 24 h immediately following the oral gavage. Intestinal permeability was assessed by measuring urinary lactulose, mannitol and lactulose:mannitol (L:M) ratio. Dietary Thr did not have any effect ($P > 0.05$) on lactulose, mannitol or L:M ratio. A significant fibre \times period ($P < 0.01$) interaction was observed, where lactulose recovery and L:M ratio was increased during the ISS period in LF fed pigs but not in HF fed pigs. Dietary Thr or period did not affect total fecal mucin output, however, HF fed pigs had a higher fecal mucin output compared to LF fed pigs ($P < 0.05$). In experiment 2, 128 pigs (22.6 ± 1.6 kg initial body weight) were housed in groups of 4 pigs/pen and were randomly assigned to dietary treatments of either a low (**LF**; 13% total DF) or high fibre (**HF**; 20% total DF) with either a standard (**STD**; 0.65% SID) or supplemental (**SUP**; 0.78% SID) level of Thr. The experiment protocol was initiated for 28 d (7 d unchallenged period and 21 d post- ST inoculation). On d 7 post-inoculation, one pig/pen was euthanized and tissue and digesta samples were collected for RT-qPCR, morphological and volatile fatty acid (**VFA**) analysis. Fecal samples were collected from 2 pigs in each pen 2 d before and on d 4 post-inoculation for fecal mucin analysis. Inoculation with ST increased ($P < 0.05$) fecal mucin output compared to the unchallenged period. There was an interaction ($P < 0.05$) between fibre and Thr on total fecal mucin output, where SUP Thr increased fecal mucin output in the HF-fed pigs. Feeding HF increased ($P < 0.05$) acetate and propionate concentration in the cecum and increased acetate and butyrate concentration ($P < 0.05$) in the colon. No effect ($P > 0.05$) of either Thr or fibre on expression of gene markers of barrier function was observed except for a tendency ($P = 0.06$) for

increased expression of MUC2 with HF diets. There was no effect of diet on villus height, crypt depth, or villus:crypt ratio ($P > 0.05$), however, there was higher goblet cell number with HF diet ($P < 0.05$). In conclusion, DF appears to improve barrier function, as indicated by reduced L:M ratio, through an increase in mucin production capacity (i.e., goblet cell numbers, MUC2 gene expression) and secretion (i.e., fecal mucin output). The lack of effect of dietary Thr provides further evidence that mucin production in the gut is conserved. Dietary Thr may therefore be limiting for growth under conditions of increased mucin secretion, such as feeding high DF and during an enteric pathogen challenge.

7.2 Introduction

The intestinal epithelium consists of a single layer of cells which creates a separation between the intestinal lumen and the underlying tissues of the body and plays a key role in responding to changes in the luminal environment (Wells et al., 2010; 2011; Zhang et al., 2015). The epithelial layer also functions as a selective barrier allowing for nutrient absorption while preventing toxins, bacteria, and other foreign compounds from entering body circulation. Physical and chemical factors present in the intestinal lumen contributes to the functions of the intestinal barrier (Farhadi et al., 2003; Magalhaes et al., 2007). In commercial swine production, pigs are exposed to environmental and dietary factors that negatively impact barrier function, affecting their production performance and efficiency (McGuckin et al., 2009; Chen et al., 2013; Che et al., 2014). Indeed, a compromised barrier is thought to predispose animals to enteric pathogens and luminal toxins, ultimately inducing inflammation, lowering feed intake and efficiency of feed utilization for growth (Ewaschuk et al., 2011; Zhu et al., 2013). Mucus is a glycoprotein secreted by goblet cells that acts to protect the intestinal epithelium from mechanical, chemical and bacterial injuries (Corfield et al., 2000). Mucus is a major contributor to improved intestinal barrier function, facilitating an unstirred water layer and an environment that limits direct contact of luminal antigens with the epithelium.

Dietary fibre (**DF**) is thought to have both direct and indirect effects on intestinal health and barrier function, including alterations in mucus secretion and cell proliferation as well as changes in the luminal environment as a result of fermentation metabolites (Blank et al., 2012;

Chen et al., 2013; Molist et al., 2014; Saqui-Salces et al., 2017). However, there are associated negative effects of feeding high DF on endogenous AA losses. Losses of Thr are particularly high because mucus contains high amounts of mucin, a Thr rich glycoprotein (Faure et al., 2002; Dharmani et al., 2009). Since mucin is largely resistant to digestion, increased secretion of mucus will result in high endogenous losses of Thr (Libao-Mercado et al., 2007; Blank et al., 2012). Although mucin secretion is conserved and prioritized (Munasinghe et al., 2017), there is evidence that mucin secretion might be sensitive to dietary Thr concentration (Faure et al., 2005). Previous studies have reported improved intestinal barrier function, increased goblet cell density and increased expression of MUC2 mRNA in pigs and chickens (Wang et al., 2010, Azzam et al., 2011b; 2012) when Thr supply was above dietary requirements for growth.

Therefore, the objective of this study was to characterize the independent and interactive effects of DF and Thr on intestinal barrier function and gut health by measuring markers related to intestinal health and barrier function in pigs during periods of immune stress. It was hypothesised that high DF will increase mucus secretion in the gut and improve intestinal barrier function, regardless of the immune status of growing pigs.

7.3 Materials and Methods

The experimental protocols were approved by the University of Saskatchewan's Animal Research Ethics Board under protocols 20160107 and 20180123 and followed Canadian Council on Animal Care guidelines (CCAC, 2009).

7.3.1 Experimental Procedures

Experiment 1

As previously reported by Wellington et al. (2018), a total of 90 growing barrows (Camborough Plus \times C3378: PIC Canada Ltd.) with initial body weight of 20.5 ± 0.75 kg were individually housed in metabolism crates (1.4 m \times 1.5 m) in a temperature-controlled room at 20 ± 2 °C at the Prairie Swine Centre, Inc. (Saskatoon, SK, Canada). Pigs were randomly assigned

to 1 of 10 dietary treatments over 9 blocks with a total of 10 pigs per block and 9 pigs per treatment. The dietary treatments were arranged as a 2×5 factorial in a randomized complete block with factors of fibre level [high fibre (**HF**) or low fibre (**LF**)] and threonine level (0.49, 0.57, 0.65, 0.73 and 0.81% SID). The HF diets were formulated with commercial feed ingredients by partly replacing corn in the LF diet with 5% wheat bran and 10% sugar beet pulp. The fibre levels were representative of typical fibre ingredients used commercially in North America. The experiment lasted for 16 d with an 8-d adaptation period followed by two 4 d collection periods, a pre-immune system stimulation (pre-**ISS**) period and an ISS period. Immune system stimulation was achieved by intramuscular injection with *E. coli* lipopolysaccharide (LPS; O55:B5, Sigma Aldrich, Oakville, ON, Canada) at an initial dose of 30 µg/kg BW and repeated after 48 h at a 15% increase in dosage (Rakhshandeh and de Lange, 2012). On d 4 of each period, after an overnight fast, pigs were orally dosed using temporary gastric tube (18FR, MED-RX, Canadian Hospital Specialties, Ltd., Oakville, ON, Canada) with a lactulose solution (0.67 g/mL Apo-Lactulose, Apotex Inc. Toronto, ON, Canada) and a mannitol solution (0.43 g/mL Mannitol, Sigma Aldrich, Oakville, ON, Canada) to provide 0.5 g and 0.3 g/kg BW lactulose and mannitol, respectively. Following the oral gavage, urine was collected over a 24 h period from each pig. The total urine collected was weighed and a 10% aliquot was sampled and stored at -20 °C for analysis later. Fresh fecal samples were obtained from individual pigs each day during both pre- and post-ISS periods and stored at -20 °C. At the end of the collection daily fecal samples were pooled for each pig and stored at -80 °C. The fecal samples were freeze dried, pooled and ground for each pig and mixed thoroughly before sub-sampling for fecal mucin analysis.

Experiment 2

As previously reported by Wellington et al. (2019b), a total of 128 pigs (Camborough Plus × C3378; PIC Canada Ltd.) of 22.6 ± 1.6 kg initial BW were housed in groups of 4 pigs/pen on solid floors lined with rubber mats in a temperature-controlled room (22 ± 1 °C). Pens were randomly assigned to 1 of 4 dietary treatments which consisted of a low fibre (**LF**; 13% total DF) or high fibre (**HF**; 20% total DF) diet with either a standard (**STD**; 0.65% SID) or supplemental (**SUP**; 0.78% SID) Thr level. The HF diets were formulated by partly replacing corn in the LF diet with 5% wheat bran and 10% sugar beet pulp and represented typical commercial diets. The

experiment lasted for a total of 28 d and consisted of a 7-d adaptation period (unchallenged) and 21 d post-*Salmonella typhimurium* (ST) inoculation period. On d 0 of the challenge period, pigs were orally inoculated twice within 4 h with a saline solution containing 2.3×10^9 CFU/mL of ST selected to be resistant to the antibiotics Novobiocin (Nov+) and Nalidixic acid (Nal+). Fresh fecal grab samples were collected from 2 pigs in each pen -2 d before and on d 4 post-ST challenge and stored at -20 °C and subsequently at -80 °C before freeze-dried and ground and mixed completely before subsampling for fecal mucin analysis. On d 7 post-inoculation, one pig/pen representing the average pen BW was humanely euthanized by penetrating captive bolt followed by exsanguination. Subsequently, intestinal tissue (ileum, cecum, and colon) were sampled and snap-frozen in liquid nitrogen and later stored in -80 °C for RNA isolation and RT-qPCR. Additionally, ileal tissue samples were stored in 10 % buffered formalin solution (Thermo fisher Scientific Ltd., Toronto, ON, Canada) for tissue morphological analysis. Digesta samples (ileum, cecum, and colon) were collected into 15 ml tubes and stored at -80 °C for volatile fatty acid (VFA) analysis.

7.3.2 Analytical Procedures

7.3.2.1 In Vivo Barrier Permeability Analysis (Experiment 1)

Urinary analysis for lactulose and mannitol was completed at the National Research Council (Saskatoon, SK, Canada) using ion chromatography based on the procedure of Hurum and Rohrer (2016). Briefly, the urine samples were diluted (1:100) with deionized water and the mixture inverted several times. An aliquot of 1 mL of the sample mixture was transferred into 1.5 mL polypropylene injection vials. Lactulose and mannitol concentration was analyzed on a Dionex ICS-3000 ion chromatography system (Thermo Scientific, Sunnyvale CA, USA) using Chromeleon software (version 6.80 SR10, build 2818) with a Dionex CarboPac MA1 4 x 50 mm guard followed by a Dionex CarboPac MA1 BioLC Analytical 4 x 250 mm column (Thermo Scientific, Sunnyvale CA, USA). The mobile phase was 480 mM NaOH at a flow rate of 0.4 mL/min. The detector was programmed to quantify using the calibration curves run with the samples to yield the amount of lactulose or mannitol ($\mu\text{g/mL}$). Standards were prepared and analyzed with each batch of urine samples.

7.3.2.2 Total Fecal Mucin Analysis (Experiment 1 and 2)

Fecal mucin was analyzed according to methods described by the kit manufacturer (Fecal mucin assay kit, Catalog #CSR-FFA-MU-K01E CosmoBio, Ltd. Tokyo, Japan). Briefly, fecal samples were freeze-dried and ground, and 100 mg of fecal sample was weighed into 2 mL tubes and 1 mL of buffer was added before the samples were vortexed and heated at 95 °C to denature bacterial glycosidase. The samples were then centrifuged at $20,000 \times g$ at 4 °C and the supernatant was harvested and treated with additional buffers and an enzyme solution to hydrolyse the starch. Following that, 615 μ L of ethanol was added to the mixture and stored overnight at -20 °C. After overnight storage, samples were centrifuged at $20,000 \times g$ at 4 °C for 15 min to precipitate the N-acetylgalactoseamine. The supernatant was discarded, leaving the pellet, which was re-suspended in a buffer solution and reagents were added and heated at 100 °C for 30 min. The final sample mixture was cooled to room temperature and 100 μ L was transferred into 96 well black plates, excited at 336 nm wavelength and the emission was measured at 383 nm wavelength using a fluorescent plate reader (SynergyTM Multi-Mode Reader, BioTex Instruments, Inc., Vermont, USA). The emission values were recorded and plotted on a standard curve of N-acetylgalactoseamine provided in the kit to determine fecal mucin concentration (mg/g of feces on DM basis). The total fecal mucin output (mg/d) was then calculated in both experiments by multiplying the fecal mucin concentration (mg of mucin/g of feces DM basis) measured by the kit and the total fecal output based on dry matter digestibility as determined previously (Wellington et al., 2018) and the feed intake as measured in experiment 1 and 2.

7.3.2.3 Ileal Morphology and Goblet Cell Counts (Experiment 2)

Ileal tissue samples were obtained 15 cm from the ileocecal junction and immediately fixed in 10% buffered formalin. The fixed intestinal segments were embedded in paraffin and sectioned for intestinal morphology (Prairie Diagnostic Services, Saskatoon, SK). Briefly, sections of the tissue were deparaffinized in xylene, rehydrated and stained with haematoxylin and eosin. Slide images were measured at 10 \times magnification using an Axio Star Plus light microscope (Axio Scope A1; Carl Zeiss Gottingen, Germany). The villus height and crypt depth of each tissue were measured using the AxioVision Rel 4.8 software (Carl Zeiss Canada Ltd., Toronto, ON) on a

minimum of 10 well-oriented villi and their corresponding crypts per sample. Goblet cell counts were determined by preparing tissue samples as indicated above and staining with Alcian Blue and Periodic Acid Schiff as previously described (Sweich et al., 2019). The slides were then viewed under a light microscope at $10\times$ magnification (Axio Scope A1; Carl Zeiss Gottingen, Germany). For each tissue sample, 10 well-oriented villi were selected, and goblet cells were counted in a region within 100 μm length of the villi.

7.3.2.4 Volatile Fatty Acid (VFA) Analysis (Experiment 2)

Volatile fatty acid (VFA) analysis followed the procedure by Khorasani et al. (1993) and Lenahan et al. (2010). Briefly, digesta samples (ileum, cecum and colon) were diluted with 25% metaphosphoric acid at a 2:1 ratio (w/v). Samples were centrifuged at $12,000\times g$ for 10 min and the supernatant was collected into 2 mL centrifuge tubes and further centrifuged at $16,000\times g$ for 10 min. Following that, the supernatant was collected and filtered through 0.45 μm PVDA filter (Fisher Scientific, Hampton, New Hampshire, USA) into 1.5 mL tubes. An internal standard (4.56 $\mu\text{mol/mL}$ isocaproic acid in 0.15 mol/L oxalic acid) was added at 0.2 mL to 1 mL of the filtered sample supernatant and inverted to mix thoroughly. The volatile fatty acids were determined on an Agilent 6890 gas chromatograph with a flame ionization detector (Agilent Technologies, Santa Clara, California, USA) and a capillary column ZB-FFAP (30 m length \times 0.32 mm width \times 0.25 μm film thickness; ZEBRON, Phenomenex, Torrance, California, USA). The initial oven temperature was set at 90 $^{\circ}\text{C}$ and a hold time of 0.1 min, then followed by the 1st ramp; 10 $^{\circ}\text{C}$ per minute which increased until 170 $^{\circ}\text{C}$ with a hold time of 6 sec. The 2nd ramp was 20 $^{\circ}\text{C}$ per minute up to 230 $^{\circ}\text{C}$ with and a hold time of 2 min. Hydrogen gas was used for the FID and helium gas was used as a carrier.

7.3.2.5 RNA Isolation cDNA Synthesis and RT-qPCR Analysis (Experiment 2)

Tissue samples stored at -80 °C were ground in liquid nitrogen with mortar and pestle and total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration and quality were determined using a spectrophotometer (NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific Inc., Delaware, USA) with optical density ratio (260:280) between 1.8 and 2.0. The integrity of RNA was then assessed by gel electrophoresis. Reverse transcription was carried out using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA) with random hexamer primers. Each 20 µL of reaction mix contained 0.8 µL of 10µM primer concentration for each forward and reverse primer, 6.4 µL of nuclease-free water, 10 µL of EVA Green supermix (Bio-Rad Laboratories, CA, USA) and 2 µL (2 ng/qPCR reaction) of template cDNA. Standard curves were made for each gene using a 5-fold serial dilution of pooled cDNA samples from all experimental treatments. PCR efficiency between 90-110% were accepted. All genes (Table 7.1) were analyzed with a standard dilution series prepared from cDNA on the same plate and the starting quantities (recorded as arbitrary values) calculated for each gene on each plate. Arbitrary values for genes of interest were normalized using the mean of the arbitrary values for GAPDH and RPL19 which were confirmed to be not affected by treatment.

7.3.3 Statistical Analyses

Data were tested for normality and outliers were verified using the Shapiro-Wilk test and the studentized respectively (PROC UNIVARIATE, SAS 9.4, SAS Institute Inc., Cary, NC). In experiment 1, all data were analyzed as a 2×2×5 factorial in a randomized complete block design (PROC MIXED, SAS 9.4, SAS Institute Inc., Cary, NC). The model included main effects of (a) fibre level [High or Low DF], (b) period [ISS or Pre-ISS] (c) threonine level [0.49, 0.57, 0.65, 0.73 and 0.81% SID] and their interactions and block as a random effect. In experiment 2, VFA, qPCR, and ileal morphology and goblet cell samples were analyzed as 2×2 factorial arrangement in a randomized complete block design. The model included the main effects of (a) fibre [High or Low DF] and (b) threonine [STD Thr or SUP Thr] and their interaction and block as a random effect. Data for the fecal mucin output were analyzed as 2×2×2 factorial arrangement in a randomized

complete block design. The model included the main effects of (a) fibre [High or Low DF] and (b) threonine [STD Thr or SUP Thr] and (c) period (pre-ST inoculation and post-ST inoculation) and their interactions and block as a random effect. Significant differences were determined at $P < 0.05$ and a trend toward significance considered at $P \leq 0.10$ and when significance was observed, the means were separated by the least significant difference method (LSD method; SAS 9.4, SAS Institute Inc., Cary, NC).

Table 7.1 Primers used in quantitative PCR analysis¹

Gene	Forward (5' to 3')	Reverse (5' to 3')	AT (°C)	NCBI accession number
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG	60	AF_435591
MUC2	ACCCGCACTACGTCACCTTC	GGCAGGACACCTGGTCATTG	62	BX671371
CLDN4	CAACTGCGTGGATGATGAGA	CCAGGGGATTGTAGAAGTCG	60	NM_001161637.1
GAPDH	CTTCACGACCATGGAGAAGG	CCAAGCAGTTGGTGGTACAG	63	AF017079
ZO-1	ACGGCGAAGGTAATTCAGTG	CTTCTCGGTTTGGTGGTCTG	60	XM_003353439.2
IL8	TCCTGCTTTCTGCAGCTCTC	GGGTGGAAAGGTGTGGAATG	62	NM_213867
Casp3	AGAGGGGACTGCTGTAGAACT	CCGTCTCAATCCCACAGTCC	59	NM_214131.1

¹GAPDH = glyceraldehyde 3-phosphate dehydrogenase; RPL19, ribosomal protein-L19; ZO1 = Zonula Occludin -1; MUC2 = Mucin-2; CLDN-4 = Claudin-4; IL8 = Interleukin-8; Casp3 = Caspase-3, AT = annealing temperature.

7.4 Results

7.4.1 *In Vivo Barrier Permeability Measurements (Experiment 1)*

There was no Thr effect on lactulose or mannitol recovery or lactulose:mannitol (**L:M**) ratio. As shown in Fig 7.1, significant period \times fibre interaction ($P < 0.01$) was observed on lactulose recovery and L:M ratio. When no immune stimulation was present, lactulose recovery and L:M ratio was not different between LF and HF-fed pigs, however following ISS, lactulose recovery and L:M ratio was increased in LF-fed pigs, but not in HF-fed pigs. The recovery of mannitol was not significantly affected ($P > 0.05$, Fig 7.1B) by either Thr, fibre or period, with no interaction.

7.4.2 *Total Fecal Mucin Output*

In experiment 1, the total fecal mucin output was determined in feces of pigs during pre-ISS and ISS periods and data are presented in Fig 7.2. Neither dietary Thr nor ISS by LPS had an effect on total fecal mucin output ($P > 0.05$), however, total fecal mucin output was increased in the HF fed pigs ($P < 0.05$).

In experiment 2, before ST inoculation a lower ($P < 0.01$) total fecal mucin output (301.8 mg/d) was observed compared to total fecal mucin output (588.9 mg/d) post ST inoculation (Fig. 7.3A). A fibre \times Thr interaction (Fig. 7.3B; $P = 0.03$) on total fecal mucin output was observed where Thr supplementation increased total fecal mucin output to a greater extent in pigs fed HF diet compared to LF-fed pigs.

7.4.3 *Ileal Morphology and Goblet Cell Numbers (Experiment 2)*

There were no effect of fibre, Thr, an interaction on the villus height, crypt depth, or villus height:crypt depth ratio ($P > 0.05$) in ileal tissue samples of pigs 7 d post-ST inoculation (Table 7.2). No effect of Thr ($P > 0.05$) was observed in goblet cell number, however, goblet cell numbers were increased ($P = 0.04$) in HF-fed pigs compared to the LF-fed pigs (Fig 7.4).

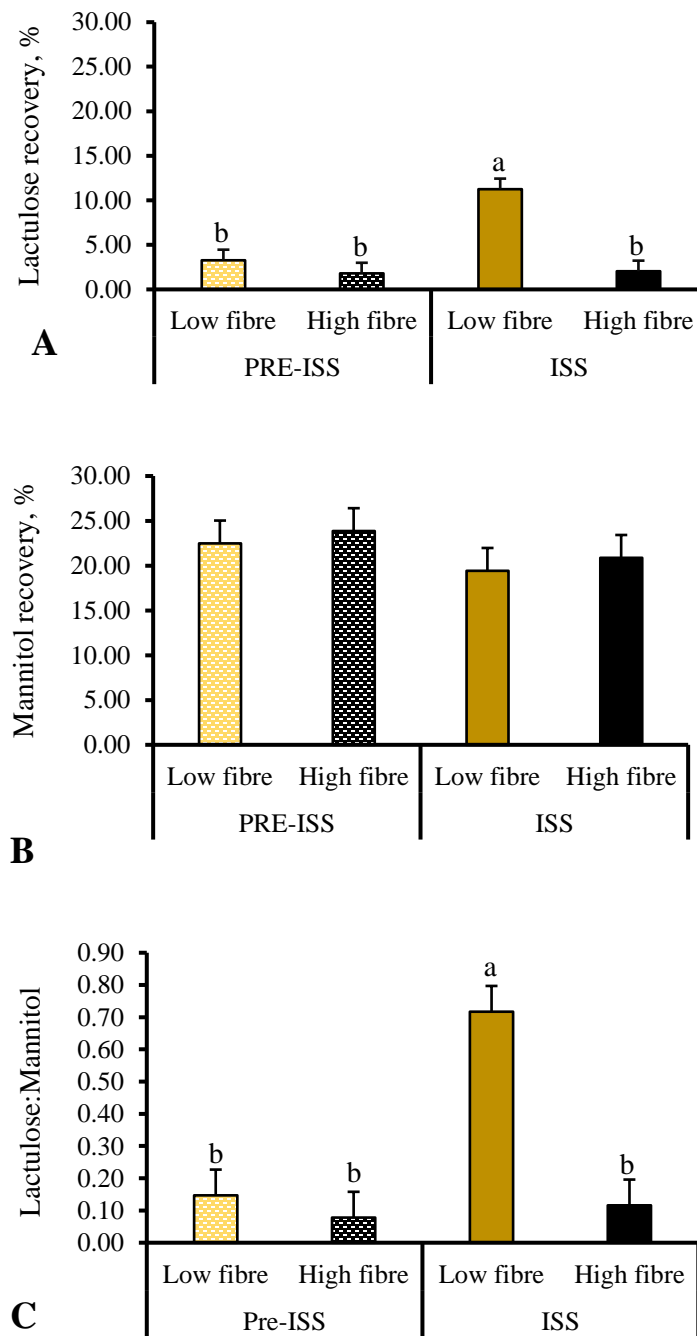


Figure 7.1 Urinary lactulose (A), mannitol (B) and lactulose:mannitol ratio (C) in *E. coli* lipopolysaccharide challenged and unchallenged pigs fed either high or low fibre diets with graded dietary threonine levels. A total of 9 replicate pigs/treatment were used in the analysis.

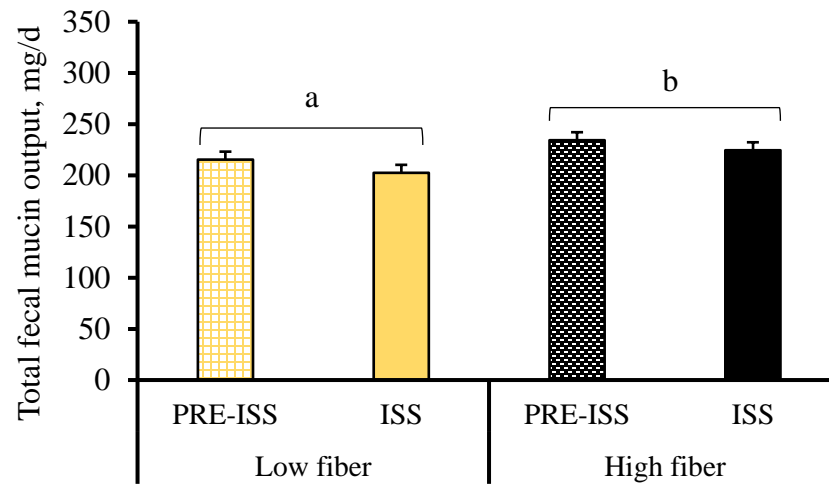


Figure 7.2 Total fecal mucin output (mg/d) in LPS challenged and unchallenged pigs fed high or low fibre with graded dietary threonine. Total fecal mucin output was estimated using determined mucin concentration in feces and estimated total fecal output based on previously determined dry matter digestibility (Wellington et al., 2018). A total of 9 replicate pigs/treatment were used in the analysis. There was a significant effect of fibre ($P < 0.05$) with no effect of Thr or period ($P > 0.05$) on total fecal mucin output.

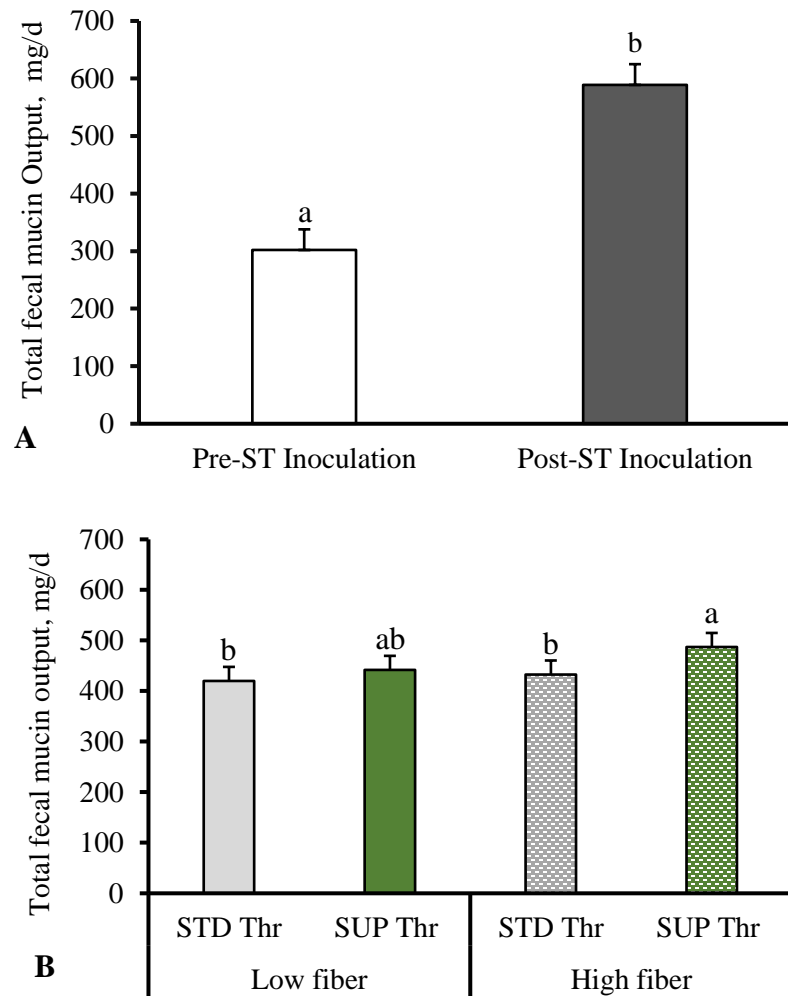


Figure 7.3 Total fecal mucin output (mg/d) 2 d before and 4 d post-*Salmonella typhimurium* inoculation in pigs fed high or low fibre diets with either standard or supplemental dietary threonine. Total fecal mucin output was estimated using determined mucin concentration in feces and estimated total fecal output based on previously determined dry matter digestibility (Wellington et al., 2018). A total of 8 replicate pigs/treatment were used in the analysis. Total fecal mucin output was higher post-ST inoculation ($P < 0.01$) compared to output pre-inoculation (**A**). There was a significant fibre \times Thr interaction ($P < 0.05$) on total fecal mucin output (**B**).

Table 7.2 Ileal morphology in pigs' 7-d post-*Salmonella typhimurium* inoculation¹

<i>Item</i>	Low fibre		High fibre		SEM⁴	<i>Fibre</i>	<i>P - value</i>	
	STD² Thr	SUP³ Thr	STD Thr	SUP Thr			<i>Thr</i>	<i>Fibre × Thr</i>
Villus height, µm	433.9	438.8	446.4	434.2	25.6	0.867	0.859	0.697
Crypt depth, µm	268.7	290.9	313.5	292.7	17.6	0.157	0.963	0.188
VH:CD5	1.69	1.59	1.46	1.57	0.10	0.207	0.998	0.271

¹Total of 8 replicate pigs/treatment (n=8/treatment).

²STD Thr, Standard Thr (0.65 % standardized ileal digestible).

³SUP Thr, Supplemental Thr (0.78 % standardized ileal digestible).

⁴SEM, Standard error of the mean.

⁵VH:CD, villus height:crypt depth.

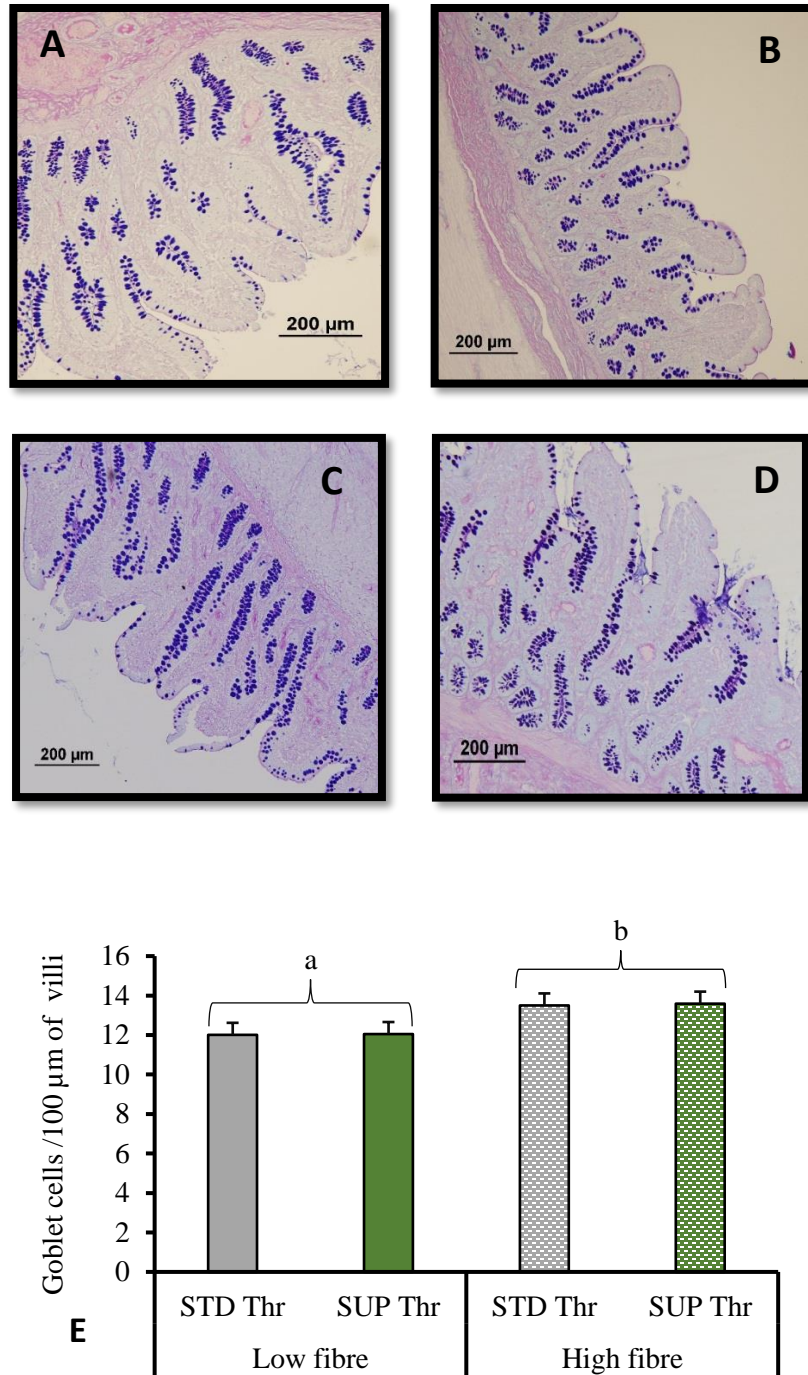


Figure 7.4 Goblet cell count (n/100 µm length of villi) of pigs challenged with *Salmonella thyphimurium*. Figure 7.4E shows a significant ($P = 0.04$) increase in goblet cell number with high fibre diets. A total of 8 replicate pigs/treatment were used in the analysis. Figure 7.4A and 7.4B shows high fibre with STD Thr and low fibre with STD Thr respectively. Figure 7.4C and 7.4D shows high fibre with SUP Thr and low fibre with SUP Thr respectively.

7.4.4 Volatile Fatty Acid Concentration (Experiment 2)

Results for VFA concentration in digesta are presented in Table 7.3. In the ileal digesta, there were no effects of fibre, Thr or their interaction on the concentration of VFA. In the cecal digesta, HF increased ($P < 0.05$) the concentration of acetate, propionate and the total VFA, but had no significant effect on butyrate concentration ($P > 0.05$). Interestingly, we observed that the total VFA concentration, primarily influenced by the acetate concentration in cecal digesta increased ($P < 0.01$) with SUP Thr.

In the colonic digesta, we observed no significant Thr effect ($P > 0.05$) on VFA concentration. However, HF increased ($P < 0.01$) colonic concentration of acetate, with a tendency ($P = 0.08$) to increase butyrate concentration and no effect ($P > 0.05$) on propionate concentration. Total VFA concentration in the colonic digesta was higher ($P < 0.05$) in the HF fed pigs than in LF fed pigs.

7.4.5 Gene Expression of Markers for Intestinal Barrier Function (Experiment 2)

Gene expression is presented in Fig 7.5 as relative expression (arbitrary units) for ileal and colonic tissue samples. In the ileum, there was a tendency for greater expression of MUC2 ($P = 0.06$) and IL8 ($P = 0.10$) with the HF diet (Fig 7.5A). There was no significant effect of Thr or interaction of Thr and fibre on the ileal expression of selected genes. There were no treatment effects on the expression of marker genes in the colon ($P > 0.05$; Fig 7.5B).

Table 7.3 Effect of fibre and threonine on volatile fatty acid concentration in digesta of pigs challenged with *Salmonella typhimurium*¹

	Low fibre		High fibre		SEM ⁴	<i>P-value</i>		
	STD ² Thr	SUP ³ Thr	STD Thr	SUP Thr		<i>Fibre</i>	<i>Thr</i> ⁵	<i>Fibre</i> × <i>Thr</i>
Cecum, µmol/g								
Acetate	58.31	66.29	64.41	87.33	4.71	<0.01	<0.01	0.09
Propionate	45.02	48.52	55.56	63.86	5.91	<0.05	0.35	0.70
Butyrate	25.56	29.47	21.47	26.16	2.76	0.25	0.18	0.90
Total VFA ⁶	128.9	144.26	141.44	177.35	9.37	<0.05	<0.01	0.26
Colon, µmol/g								
Acetate	46.39	45.99	67.69	57.06	7.37	<0.01	0.32	0.35
Propionate	25.65	25.35	29.01	27.41	4.18	0.47	0.80	0.86
Butyrate	13.34	15.24	18.32	18.42	2.75	0.08	0.66	0.69
Total VFA	85.38	86.65	115.02	102.87	12.93	<0.05	0.61	0.53
Ileum, µmol/g								
Acetate	13.76	14.21	14.86	12.64	3.66	0.92	0.71	0.58
Propionate	0.11	0.31	0.43	0.54	0.03	0.15	0.38	0.76
Butyrate	0.85	0.70	0.41	0.76	0.03	0.47	0.70	0.33
Total VFA	14.68	15.21	15.69	13.95	4.08	0.96	0.82	0.67

¹ Total of 8 replicate pigs/treatment (n=8/treatment).²STD Thr, Standard threonine³SUP Thr, Supplemental threonine⁴SEM, Standard error of the mean⁵Thr, Threonine⁶VFA, Volatile fatty acid.

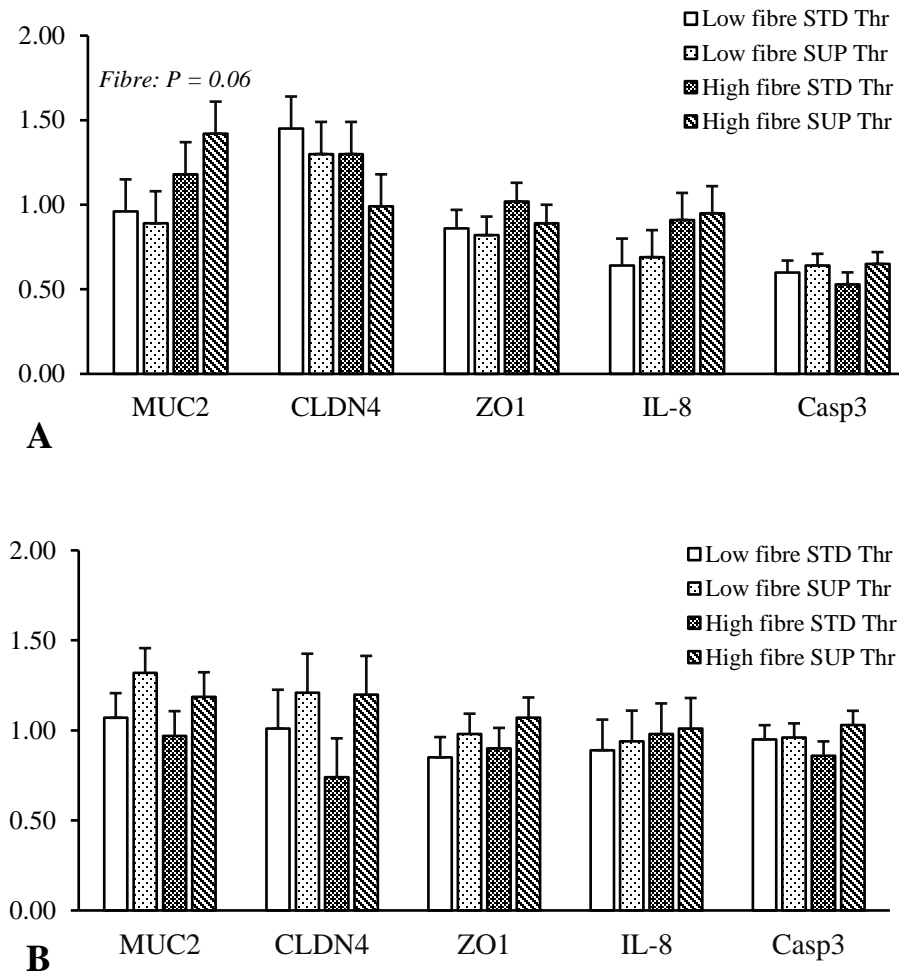


Figure 7.5 Ileal tissue (**A**) expression of marker genes for intestinal barrier function and colonic tissue (**B**) expression of marker genes for intestinal barrier function. A total of 8 replicate pigs/treatment were used in the gene expression analysis (n=8/treatment)/ ZO1 = Zonular Occludin -1; MUC2 = Mucin-2; CLDN-4 = Claudin-4; IL8 = Interleukin-8; Casp3 = Caspase-3.

7.5 Discussion

Due to the increase in co-product use in swine diets, there is increased research interest in investigating how fibre affects nutrient digestibility, animal performance, and intestinal health and barrier function. Previous work has demonstrated both direct and indirect roles of fibre on intestinal health and barrier function, largely associated with the modulatory effect of fibre on microbial environment and the subsequent production of SCFA which affects intestinal health and barrier function (Bach Knudsen et al., 2012; Chen et al., 2013; Heinritz et al., 2016). Similarly, immune challenge has been shown to affect intestinal health and barrier function as demonstrated by reduced villi height and crypt depth and increased mast cells in LPS challenged weaned pigs (Zhu et al., 2013). In addition, enteric *E. coli* challenge has been shown to disrupt tight junction assembly and destroy intestinal barrier function in rats (Shifflett et al., 2005). On the other hand, amino acids are known to play physiological roles as regulators of important functions, apart from participating in protein synthesis (Wu, 2010). For example, Thr is reported to play a role in maintaining mucosal integrity and barrier function by supporting mucin secretion (Mao et al., 2011; Sweich et al., 2019). As such, dietary Thr content has been reported to affect mucin dynamics (Kansagra et al., 2003; Faure et al., 2005; Puiman et al., 2011) and generally support intestinal barrier function and gut health (Zhang et al., 2016).

The aim of the present study was therefore, to evaluate the independent and combined effects of dietary fibre and Thr on intestinal health and barrier function using different conditions of immune challenge, including a systemic immune challenge using intravenously administered *E. coli* LPS or enteric challenge with oral *Salmonella typhimurium*. In the first experiment, we used the L:M absorption test to estimate barrier permeability (Deng et al., 1999; Wijtten et al., 2011a; Huygelen et al., 2012) as the design allowed for total urine collection which has been reported to improve the accuracy of this method (Wijtten et al., 2011a; Wijtten et al., 2011b; Huygelen et al., 2012; Li et al., 2018). In experiment 2, tissue analysis for histology and gene expression were used to assess barrier function. In both experiments, fecal samples were analyzed for fecal mucin concentration and further estimation of total fecal mucin output based on previously determined dry matter digestibility and fecal output (Wellington et al. 2018).

In experiment 1, we evaluated the effect of systemic LPS injection as an immune stimulatory agent on barrier function and further characterized the role of fibre and Thr on the intestinal barrier function with or without LPS induced immune challenge. Previous work has validated the use of lactulose and mannitol as a credible method of determining intestinal barrier permeability in pigs (Wijtten et al., 2011a; Wijtten et al., 2011b; Huygelen et al., 2012; Li et al., 2018). Movement of lactulose across the intestinal epithelium occurs only through paracellular routes whereas movement of mannitol occurs through both paracellular and transcellular pathways (Wijtten et al., 2011b). Therefore, high urinary lactulose recovery is an indicator of increased intestinal permeability (Huygelen et al., 2012). Dietary factors (e.g. fibre) could influence the absorption pathways and metabolism of both these sugars. It is assumed that both sugars will be affected equally by pre-absorptive dietary influences, however, mannitol is regarded more as a normalizing control (Wijtten et al., 2011b) and therefore reporting the L:M ratio is more indicative of response to dietary treatments.

Immune system stimulation increased the L:M ratio in LF but not HF fed pigs, which is an indication of reduced barrier function during ISS induced by LPS injection. Previously, the effect of LPS on increased intestinal permeability was reported to occur largely as a result of secondary effect mechanisms, such as oxidative stress to the intestinal epithelium (Courtois et al., 2003; Sheth et al., 2009). Observations from the current study suggest that, under conditions of immune stimulation, barrier function is reduced, but feeding high DF may provide a protective effect on the gut and contribute to improved barrier function. Since increased DF has been reported to increase mucin secretion and potentially contribute to barrier function, total fecal mucin output was measured in the same group of pigs. Measuring the output of mucin in feces provides information on the net output of mucin secreted into the gut lumen (Lien et al., 2001). The absence of an LPS induced effect on total fecal mucin output is contrary to previous studies where systemic LPS was administered (Faure et al., 2003; Faure et al., 2007). However, the measurement for mucin secretion (i.e. mucosal scrapings) used in those studies was different from fecal mucin measurement used in the present study. Hence, the lack of effect of LPS on fecal mucin is likely due to the limited direct effects of a systemic LPS induced immune stimulation on the gut epithelium. The HF diet increased the total fecal mucin output in the present study consistent with previous reports demonstrating increased ileal mucosal protein losses (e.g. mucin) (Moesar and Van Kempen, 2002; Brownlee et al., 2003; Piel et al., 2005; Libao-Mercado et al., 2007) with

increased DF. The increased mucin secretion in response to the HF diet in the present study may directly contribute to protection against paracellular lactulose transport, but also may indicate other concurrent changes in barrier function affecting transcellular permeability in response to systemic LPS. The lack of effect of ISS on mucin production provides support for the lack of additivity between HF and ISS on Thr requirements as previously reported (Wellington et al., 2018), and is likely due to the increased mucin secretion due to HF diet masking the effect of ISS on Thr requirement. Further, the effect of HF on increased mucin secretion will directly increase Thr use for mucin secretion and limits the efficiency of Thr utilization for PD, hence the observed increase in Thr requirement with the HF diet (Wellington et al., 2018).

In experiment 2, the effects of dietary fibre and Thr on intestinal health and barrier function were further evaluated in pigs challenged with an enteric pathogen (i.e., *Salmonella typhimurium*). Measuring the output of mucin in feces provides information on the net output of mucin secreted largely into the hind gut (Lien et al., 2001). The increase in total fecal mucin output with enteric ST inoculation agrees with a previous study that reported increased mucin secretion following immune stress in rats induced by live pathogenic *E. coli* (Faure et al., 2007). Although there is some information indirectly relating the impact of immune stimulation on mucus secretion in pigs (Rakhshandeh et al., 2013) and chickens (Lucke et al., 2018), the present study demonstrates direct evidence that enteric *Salmonella typhimurium* challenge increases mucus secretion. This contrasts the lack of fecal mucin output response in LPS challenged pigs reported here. *Salmonella typhimurium* challenge induced an immune response in pigs as indicated by clinical response data (rectal temperature, acute phase proteins, and *Salmonella* shedding) without any mitigating effect of dietary fibre (Wellington et al., 2019b). However, in the present study, there was a fibre by Thr interaction on fecal mucin output, which suggests that SUP Thr increased fecal mucin output in HF but not LF-fed pigs. High fibre may, therefore, be providing some mitigating effect to an enteric challenge via improved barrier function, but this was not observed with the clinical response parameters. Although it has been shown that mucin secretion is conserved and prioritized in mucus-secreting tissues in pigs (Munashinge et al., 2017), it appears that during immune stimulation the magnitude of mucin secretion response may be associated with dietary Thr availability. Similarly, this may be related to the increased mobilization of endogenous proteins (e.g. muscle) to meet the increased Thr demand that may be associated with inflammation as previously reported (Rémond et al., 2009).

The ileal morphology and goblet cell numbers were measured to ascertain the impact of dietary fibre and Thr on intestinal barrier function. No effects of fibre or Thr were observed on ileal mucosal morphology following the *Salmonella typhimurium* challenge. A previous report by Hedemann et al. (2006) suggested that feeding pigs high insoluble DF increased villi height and improved gut morphology, however, this effect may have been masked by the concurrent *Salmonella typhimurium* challenge conditions reported here. Goblet cell numbers have been used as indicators of mucus secretion capacity in pigs (Piel et al., 2005; Sweich et al., 2019) where increases in goblet cell number, most importantly in the villus indicate an increased capacity (McDole et al., 2012). The increase in goblet cell number with HF diets reported here agrees with reports from previous studies (Piel et al., 2005; Sweich et al., 2019). Different fibre sources (e.g. rye bran, oat bran) in the diet of golden hamsters were reported to increase goblet cell numbers in the intestine (Lundin et al., 1993). Further, Piel et al. (2005) fed carboxymethylcellulose, a highly viscous and non-fermentable fibre to weanling pigs and reported a 30% increase in goblet cell number. The tight junction proteins (ZO-1, CLDN4) have been reported to play significant roles in regulating paracellular permeability and barrier function, as such changes in these proteins may indicate changes in intestinal permeability and barrier function (Hu et al., 2013; Richter et al., 2014; Jiang et al., 2019). However, no significant effect of fibre or Thr on relative expression of other target genes associated with barrier function (tight junction proteins) was observed in the present study. This may be because the pigs used in this study were all challenged with *Salmonella*, as such that the response of the tight junctions to the treatments may have been masked by the effect of the ST challenge. The products of fermentation have been reported previously to improve intestinal health through mechanisms involving modification of microbial environment and composition (Chen et al., 2013; Li et al., 2018; 2019). Therefore, we measured VFA concentration in intestinal digesta, and the impact of these metabolites in relation to the expression of genes associated with intestinal health and barrier function. As expected, feeding high DF significantly increased the concentration of total VFAs in the cecum and colon digesta. Previous work in rats demonstrated that VFAs (acetate and butyrate) induce mucus secretion in rat colon (Barcelo et al., 2000; Chen et al., 2012). Indeed, we observed a trend towards increased MUC2 expression in the ileum of pigs fed HF, which is consistent with the observed increase in goblet cell numbers. Although it has been suggested that mucin dynamics are affected by dietary Thr supply in chickens

(Horn et al., 2009), we did not observe any significant effect of Thr levels on MUC2 gene expression or the expression of other target genes in the present study.

7.6 Conclusion

In conclusion, DF appears to improve barrier function, as indicated by reduced L:M ratio, through an increase in mucin production capacity (i.e., goblet cell numbers, MUC2 gene expression) and synthesis (i.e., fecal mucin output). The contribution of DF to increased barrier function may, therefore, be mediated in part by the effect of DF on increased mucus secretion in the gut. The lack of effect of dietary Thr provides further evidence that mucin production in the gut is conserved and prioritized. Hence, dietary Thr may limit growth under conditions of increased mucin secretion, such as feeding high DF and during an enteric pathogen challenge.

7.7 Acknowledgements

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CHAPTER 8
GENERAL DISCUSSION AND CONCLUSION

8.1 General Overview

Increasing feed efficiency, production performance, and profitability are the main goals for pork producers. Therefore, opportunities that seek to reduce feed cost and enhance animal production efficiency are imperative. The use of co-products as alternate feed ingredients in swine diets has increased significantly in the last decade (Woyengo et al., 2014), primarily as means of reducing feed cost, but also as a way of improving sustainability of animal agriculture by reducing the competition for feed ingredients that could be directly utilized for human food. However, there are additional challenges with increased co-product use such as increased DF in swine diets due to high DF content in many co-products. It is generally believed that high DF has a largely negative impact on production based on the reported negative effects on nutrient digestibility, availability and utilization efficiency (Stein et al., 1999; Hansen et al., 2006) resulting in reduced growth performance. However, it is important to note that DF has also been reported to have beneficial effects on gut health (Hogberg and Lindberg, 2006) and the general well-being of pigs (Lindberg, 2014). One major concern with high DF in swine diets is the effect of fibre on Thr utilization efficiency for growth (Blank et al., 2012; Mathai et al., 2016). As such, the NRC (2012) introduced an adjustment in Thr requirement for growing pigs based on the level of dietary fermentable (soluble) fibre. Since commercial pig producers are largely sourcing these high fibre co-products, it is important to evaluate Thr requirement when feeding high fibre diets.

An additional challenge facing pork producers are the recent changes in regulations regarding antibiotic use in swine production systems as growth enhancers as well as the public pressure to reduce overall antibiotic use in livestock agriculture, which may increase disease pressure in swine production systems. An increase in the incidence of sub-clinical infections and immune stressors is known to reduce production efficiency. It has been well established that immune stimulation alters AA metabolism, such that AA are redirected away from muscle growth towards prioritized functions, such as supporting an immune response (Reeds et al., 1994, Reeds and Jahoor, 2001). In current commercial production systems, the use of high DF feed ingredients and sub-clinical immune challenge conditions may be present and could be limiting factors to optimal production performance and efficiency and overall farm profitability. Therefore, harnessing effective ways of utilizing DF for its beneficial effects and reducing the negative impact on production efficiency is key. As such the studies presented in this thesis focused on quantifying

the independent and interactive effects of high DF and immune challenge on Thr requirement to maximize PD and growth performance in growing-finishing pigs. We also evaluated the effects of feeding high DF and dietary Thr levels on markers of intestinal health and barrier function in pigs during systemic (LPS) and enteric *Salmonella typhimurium* challenge.

8.2 Assessing Threonine Requirement in High DF Fed and Immune Challenged Pigs

Two experiments were conducted to quantify Thr requirements for growing pigs (25-50kg) when fed high DF with or without immune challenge. In the first experiment (Chapter 4), pigs were fed either a low DF (12% TDF) or a high DF (20% TDF) diets, with five graded levels of Thr above and below the current requirement level according to the NRC (2012) and Evonik (AminoDat® 5.0). These diets were fed to individual pigs in two periods, with and without immune system stimulation (**ISS**). Immune system stimulation was induced by repeated intramuscular injection of *E. coli* LPS. Without ISS, the Thr requirement was estimated (via quadratic regression model) at 0.68% SID in LF-fed pigs and 0.78% SID in HF-fed pigs. This observation indicates that feeding HF diets will increase Thr requirement for muscle PD and agrees with previous work that has suggested similar effects of HF on Thr requirement for PD and growth (Libao-Mercado et al., 2006; Blank et al., 2012; Mathai et al., 2016).

During the ISS period a Thr requirement of 0.76% SID was estimated to maximize PD in LF-fed pigs. This finding is important because it demonstrated for the first time the effect of ISS on Thr requirement in growing pigs (Chapter 4). This finding has been recently confirmed by McGilvray et al. (2019a), who reported a higher Thr requirement for maintenance functions and reduced marginal efficiency of Thr utilization for PD in pigs challenged with *E. coli* LPS. When pigs were fed HF diets during the ISS period, the Thr requirement was estimated at 0.72% SID, which was 8% lower than the estimate of 0.78% SID recorded when HF was fed alone without immune challenge. Altogether, these results demonstrated that HF and ISS independently increased the Thr requirement for PD but that these effects were not additive. Since the estimate of Thr requirement with high DF diets was greater than NRC (2012) requirements, we conducted a follow-up study (Chapter 5) to confirm the Thr requirement in group-housed pigs fed the same HF diets as reported in Chapter 4 and based on growth performance (i.e., ADG, G:F). After the 28-d growth performance trial, the estimated Thr required to maximize ADG was 0.76% and

0.80% SID based on the linear broken-line model and quadratic model, respectively. This observation was in close agreement with the previously determined Thr level to maximize PD in Chapter 4. The slight difference in the estimates (0.78% vs 0.80%) could be due to the response parameters used (i.e., PD vs. ADG) and the fact that a mixed-sex was used in the growth performance study whereas only barrows were used in the N-balance study. Regardless of the measure used to estimate Thr requirement, both studies resulted in greater Thr requirement than suggested by NRC (2012) according to the fermentable fibre inclusion in the swine diets.

Previous work has reported a reduced efficiency of N utilization with increased DF (Schulze et al., 1995; Hogberg and Lindberg, 2006) and that the amount of Thr losses and, therefore, the level of increase in Thr requirement, is dependent on the fibre source and concentration in the diet (Blank et al., 2012). The differences between the current estimates of Thr requirement with high DF compared to NRC (2012) may be due to the different fibre sources used, as a mixture of soluble and insoluble fibre was fed to pigs in the current studies while NRC (2012) only accounts for soluble DF content. Therefore, the results reported in Chapters 4 and 5 demonstrated that HF increased Thr requirement for PD and growth performance and further quantified the level of Thr required based on the fibre level fed (15% inclusion). The current studies provide additional knowledge to previous work that reported the effects of feeding high DF diet on Thr utilization efficiency (Schulze et al., 1995; Chen et al., 2015; Blank et al., 2012). Furthermore, the present study demonstrated the impact of systemic immune stimulation on Thr utilization and quantified for the first time the impact LPS induced immune stimulation on Thr requirement in growing pigs. We hypothesized an interactive effect between high DF and ISS on Thr requirement for PD. There was, however, no interaction, suggesting a non-additive effect of high DF and ISS on Thr requirement. We further hypothesized that the estimated requirement for high DF or ISS should be sufficient to maintain performance when pigs are fed high DF and challenged with an enteric pathogen.

In Chapter 6, we conducted an experiment to determine if the growth performance of pigs would be maintained when Thr supply was adjusted to meet the requirement for a HF diet and ISS (i.e., 0.78% SID) during an enteric *Salmonella typhimurium* challenge which is a more commercially relevant disease model. Dietary treatments consisted of a HF or LF diet (similar diets used in chapter 4 and 5) and with either a standard dietary Thr (STD: 0.65% SID Thr)

according to NRC (2012) and AminoDat[®]5.0 (Evonik) or the previously determined supplemental Thr level (SUP: 0.78% SID Thr for high DF and ISS. In this study, feeding the HF diet reduced ADG and G:F compared to feeding the LF diet. This observation was expected and confirms previous studies that have reported the negative effects of feeding HF on growth performance (Anguita et al., 2007; Montagne et al., 2012). Also, previous reports suggest that increasing dietary Thr supply improves pig performance (Li et al., 1999; Wang et al., 2006; Trevisi et al., 2015). This was confirmed in the present study, where Thr supplementation improved growth performance regardless of DF level in both pre- and post ST inoculation periods. Since Thr was supplied in the present study to meet previously determined requirement for high DF and systemic LPS immune challenge, we expected SUP Thr would have improved growth performance in both LF and HF fed pigs to the same level. While not statistically different, the numerically lower response with SUP Thr on the growth performance of HF-fed pigs compared to LF-fed pigs suggests that the level of Thr supply in this study was insufficient to meet requirements of pigs fed HF and challenge with an enteric pathogen. This observation is in contrast with our previous work (Wellington et al., 2018) which demonstrated that both HF and ISS independently increased Thr requirement for PD but did not result in a further increase when both factors were present, suggesting a non-additive effect.

The results presented in this thesis establish that the immune challenge model (e.g., enteric pathogen, LPS challenge, sanitary conditions) will have a marked effect on the immune response and nutrient utilization patterns and confirm reports from previous studies using different models to access immune response (Pastorelli et al., 2012; van der Meer et al., 2016). Interpreting results of studies using different immune challenge models must be done with some caution. Specifically, LPS stimulation had no effect on total fecal mucin output, but *Salmonella typhimurium* inoculation increased total fecal mucin output (Chapter 7), suggesting that an enteric *Salmonella typhimurium* inoculation had a relatively higher magnitude of effect on the gut compared to a systemic LPS injection, and explains why a non-additive effect of HF and ISS on Thr requirement was observed in Chapter 4, but in Chapter 6, where an enteric challenge model (*Salmonella typhimurium*) was used, it appeared that there was an additive effect of HF and immune challenge on Thr utilization for growth was observed.

A limitation of this research was the absence of a direct determination of Thr requirement during the enteric disease challenge study. We nonetheless employed the approach of utilizing the previously determined Thr requirement during the LPS induced ISS period to determine pig performance under the enteric disease challenge. The results indicated that Thr requirement may be higher with high DF and enteric disease challenge. This further demonstrates that the disease challenge model employed in assessing AA requirement will have a critical impact on the estimate measured and as such, caution must be taken in interpreting studies reporting the effect of immune challenge on AA requirements. Based on this observation, we recommend further studies to quantify Thr requirements during an enteric disease challenge by using a dose-response approach for the Thr levels to quantify the exact Thr requirement breakpoint value by measuring either PD or ADG as the response variable. The present research also used a combination of insoluble (wheat bran) and soluble (sugar beet pulp) sources of fibre in all the experiments, at a total of 15% dietary inclusion level. However, there is evidence that the type of fibre and the dietary inclusion level may have different physiochemical effects that may influence animal physiology and affect Thr utilization. Therefore, future studies should focus on evaluating Thr requirement in pigs fed fibre sources with different physicochemical properties either independently or in combination.

8.3 Assessing Intestinal Barrier Function in High DF Fed and Immune Challenged Pigs

The effects of high DF and immune challenge on intestinal health and barrier function were evaluated in pigs fed different dietary Thr levels (Chapter 7). There is evidence of the indirect effect of high DF on gut health, which is noted to be largely influenced by microbial metabolites from fibre fermentation. In a recent report by Schulthess et al. (2019), SCFA, particularly butyrate, was shown to induce metabolic changes in macrophages which enhances their bactericidal activities and improves host barrier function. Similarly, Chen et al. (2013) reported improved intestinal barrier function in piglets fed wheat bran and pea fibre, through a mediated mechanism involving changes in microbial composition in the gut. There are suggestions that mucus secretion is enhanced by intestinal microbes, because of the benefits derived from chemical regulation of mucin synthesis (Dharmani et al., 2009). For example, feeding *E. coli* challenged weanling pigs with a higher dietary Thr (9 g Thr/kg of feed) had increased goblet cell numbers (Trevisi et al., 2015). In addition, dietary Thr deficiency has been shown in young pigs to reduce intestinal

mucosal protein synthesis, which may affect intestinal health and barrier function (Wang et al., 2007). Based on this previous work and findings in our studies (Chapter 4 and 6) we sought to further elucidate the specific mechanisms related to the effects of DF, ISS, and Thr on intestinal health and barrier function. Using the indigestible sugar recovery test method (lactulose and mannitol) as previously described by Wijtten et al. (2011a) (Chapter 7, Experiment 1), we were able to show that during periods of immune challenge, as induced in this study by LPS injection, feeding high DF reduced lactulose recovery in urine. This observation indicates that, feeding high DF under immune challenge improved intestinal barrier function. It is thought that the mechanism for this result is due to fibre induced increase in mucus secretion, which is a significant contributor to improved barrier function. By measuring the mucin concentration in feces and relating it to the total fecal output, we were able to show that feeding high DF increased mucus secretion as indicated by a higher total fecal mucin output in the HF fed pigs compared to the LF fed pigs regardless of immune status. Fibre has been associated with increased fecal bulk, and therefore demonstrates the need to adjust for fecal mucin concentration in feces based on total fecal output. Indeed, this observation was consistent with the results from Chapter 4 showing that HF increased Thr requirement for PD, most likely due to high Thr utilization for mucin secretion. Similarly, the observed non-additive effect of LPS and HF on Thr requirement was consistent with the observations made in Chapter 7, where LPS induced a loss of barrier function in LF fed pigs but not in HF fed pigs, resulting in no further increase in Thr requirement above HF alone.

In the second experiment in chapter 7, pigs were orally inoculated with an enteric *Salmonella typhimurium* and fed HF or LF diet with STD or SUP dietary Thr. We observed that the enteric immune challenge increased fecal mucin output and by extension increased mucus secretion in the gut. This observation agrees with a previous study by Faure et al. (2007), who reported a two-fold increase in mucosal protein synthesis in response to immune stimulation in rats induced by injection of live pathogenic *E. coli* bacteria. Increased goblet cell numbers in the HF fed pigs compared to the LF fed pigs further demonstrates the effect of HF on mucus secretion in the gut. The effects of fibre and Thr show a significant interaction where HF increased total mucin output in SUP Thr fed pigs. Indeed, dietary Thr supplementation above the recommended requirement for growth has been reported to improve intestinal health and immunity in weaned pigs (Mao et al., 2014). Also, when dietary Thr supply increased, parameters of improved immunity and gut health (MUC2, occludin, and sIgA) increased in laying hens fed low crude

protein diet with crystalline AA supplementation (Azzam et al., 2017). A recent study by Saadatmand et al. (2019) evaluated the effects of fibre and Thr on intestinal morphology and immune response in broiler chickens and concluded that feeding high DF reduced broiler performance. However, when high DF was combined with dietary Thr at 10% above requirement level, there was an improvement in intestinal health as measured by increased jejunal villi height and crypt depth and higher antibody titres (Saadatmand et al., 2019). There were no significant effects of fibre or Thr on mRNA expression of selected genes related to barrier function and health, except for a tendency for HF to increase MUC2 gene expression. This observation is significant because increase MUC2 secretion could be directly related to an increase in the mucus secretion in the ileum and perhaps may be a factor which provides the protective effects of fibre on immune stimulation observed in Chapter 7 (Expt.1). However, reports on the effect of fibre on gene expression have been inconsistent. For example, mannan-oligosaccharide was shown to modulate gene expression in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV), enhancing immune response and barrier function (Che et al., 2011). Similarly, β -glucans extracted from yeast was reported to have immunomodulatory activities as reflected in changes in gene expression patterns in ileal and colonic tissues (Sweeney et al., 2012). Similar observations were reported by Li et al. (2019), who saw a fibre effect on the expression of genes related intestinal health and barrier function (CLDN1, OCLN, ZO-1) in pigs challenged with *E. coli* F18. A few studies, on the other hand, have reported no effect of fibre on the expression of genes related to intestinal health and barrier function. For example, Agyekum et al. (2015) reported no effect of feeding a high fibre diet (30% DDGS) on membrane-bound mucin genes MUC4 and MUC20. The inconsistencies in available data could partly be associated with factors relating to the experimental design, because it is important to note that pigs used in the present study (Chapter 7) for gene expression analysis were all challenged with *Salmonella typhimurium* as such the response of gene expression analysis could be partly related to the possible effects of the ST challenge.

8.4 Summary and Conclusion

The overall objective of this research was to investigate and quantify the independent and combined effect of high DF and immune system stimulation on Thr requirement in growing pigs. Further, we aimed to evaluate the effect of high DF, Thr and immune system stimulation on intestinal health and barrier function. Overall, this series of studies demonstrated that high DF increased Thr requirement for PD and growth performance (ADG and G:F). Also, this study demonstrated that LPS induced ISS, increased Thr requirement for PD. However, when high DF and ISS were combined, no additive effects on Thr requirement were observed. When the previously determined Thr requirement estimates were evaluated on growth performance using an enteric disease challenge model (*Salmonella typhimurium*), the growth performance of pigs fed SUP Thr with HF was numerically lower than SUP Thr LF-fed pigs, indicative of an additive effect of HF and enteric immune challenge on Thr requirement. Dietary fibre appears to improve barrier function, as indicated by reduced L:M ratio, through an increase in mucin production capacity (i.e., goblet cell numbers, MUC2 gene expression) and synthesis (i.e., fecal mucin output). The contribution of DF to increased barrier function may, therefore, be mediated in part by the effect of DF on increased mucus secretion in the gut. The lack of effect of dietary Thr provides further evidence that mucin production in the gut is conserved and Thr may be limiting for growth under conditions of increased mucin production, such as feeding high fibre diets and enteric pathogen challenge. The non-additive effect of LPS and HF on PD is consistent with the observed effect of LPS on barrier function. Immune system stimulation induced a loss of barrier function in LF fed pigs which is reflected as an increased Thr requirement for PD. However, no loss of barrier function was observed when ISS and HF were combined as such there was no further increase in Thr above the requirement for HF alone. Feeding HF and *Salmonella typhimurium* challenge appear to have an additive effect on Thr requirement, which contradicts the non-additive effect of HF and systemic LPS on Thr requirement. Systemic LPS and enteric *Salmonella typhimurium* did not behave the same way to impact Thr requirement. The difference between these challenge models is based on their impact on the gut. An enteric immune challenge (*Salmonella typhimurium*) as against a systemic LPS challenge, will have a greater magnitude of impact on gut mucosal protein dynamics and may increase Thr utilization to support mucosal protein synthesis to maintain gut integrity, and thereby increase dietary Thr requirement for growth. Findings from this research will be beneficial to producers, nutritionists and other swine industry players, as a

basis for continuous inclusion of fibre in swine diet for its beneficial effects on gut health and barrier function, but also consider the effects of DF and immune status of pigs when formulating for dietary Thr in order to maximise growth and improve production efficiency.

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